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THE ANTICHLAMYDIAL EFFECTS OF DRUGS USED IN CARDIOVASCULAR DISEASES

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Abstract

Chronic *Chlamydia pneumoniae* infections have been associated with cardiovascular diseases (CVD), but the treatment is difficult. Some drugs used for CVD have been found to have an inhibitory effect on the *C. trachomatis* infection, which is not considered to be associated with CVD. The purpose of this study was to investigate the effects of heparan sulfate-like glycosaminoglycans, COX inhibitors and rapamycin on the *C. pneumoniae* infection with cell culture methods.

Almost any conceivable factors may affect the results of cell cultures. This study showed the complex interaction between temperature, time and medium during the pre-treatment before inoculation. The influences of these factors on the results overlapped and interlaced. The simple washing procedure could enhance the infectivity of *C. pneumoniae* although it is generally considered to cause the loss of chlamydial EBs and sequentially decrease the chlamydial infectivity.

Although the detailed mechanisms were not studied, the results of this study showed that selective COX inhibitors and rapamycin can inhibit the infectivity of *C. pneumoniae* by inhibiting the growth and maturation, whereas heparan sulfate-like glycosaminoglycans perhaps inhibit the attachment of *C. pneumoniae* EBs onto the host cells. Recovery and repassage results showed that the growth can be only delayed by selective COX inhibitors, and it can recover to normal level once the drugs were removed. However, rapamycin inhibited the maturation of chlamydial EBs and therefore the infectivity fell down further even when the rapamycin was removed. This study also presented the variations of pathogenicity between different *C. pneumoniae* strains *in vitro*. This study is based on *in vitro* experiments with an acute infection model. Thus, any definite conclusions on the possible antichlamydial effects of the drugs tested in the treatment of cardiovascular diseases which are associated with chronic *C. pneumoniae* infections cannot be drawn on the basis of this study.

Keywords: atherosclerosis, Chlamydia pneumoniae, cyclooxygenase inhibitors, fetal calf serum, glycosaminoglycan, heparin, infectivity, inhibition, rapamycin

In Memory of my Parents

Acknowledgements

Abbreviations

ACE	angiotensin-converting enzyme
ACS	acute coronary syndromes
ARB	angiotensin receptor blocker
ATP	adenosine-triphosphate
CD14	cluster of differentiation 14
CHD	coronary heart disease
COPD	chronic obstructive pulmonary disease
COX	cyclooxygenase
CpMIP	*Chlamydia pneumoniae* MIP
CSA	chondroitin sulfate A
CVD	cardiovascular diseases
DNA	deoxyribonucleic acid
EB	elementary body
EIA	enzyme immunoassay
FCS	fetal calf serum
FKBP	FK506-binding protein
GAG	glycosaminoglycan
HL	human line
Hsp	heat shock protein
IFN-γ	interferon gamma
IL	interleukin
LDL	low-density lipoprotein
LGV	lymphogranuloma venereum
LMWH	low molecular weight heparin
LPS	lipopolysaccharide
MIF	microimmunofluorescence test
MIP	macrophage infectivity potentiator
MOMP	major outer membrane protein
mTOR	mammalian target of rapamycin
NF-κB	nuclear factor kappa-light-chain-enhancer of activated B cells
NO	nitric oxide
NSAIDs	non-steroidal anti-inflammatory drugs
Omp	outer membrane protein
PBS	phosphate-buffered saline
PCR	polymerase chain reaction

PG	prostaglandin
Pmp	polymorphic membrane protein
PPlase	peptidyl-prolyl cis-trans isomerase
RB	reticulate body
RNA	ribonucleic acid
SPG	sucrose-phosphate-glutamic acid buffer
TNF-α	tumor necrosis factor alpha

List of original publications

This thesis is based on the following articles, which are referred to in the text by their Roman numerals.

I   Yan Y, Silvennoinen-Kassinen S, Leinonen M & Saikku P (2005) Methodological aspects affecting the infectivity of *Chlamydia pneumoniae* in cell cultures *in vitro*. J Microbiol Methods 61: 127–130.

II   Yan Y, Silvennoinen-Kassinen S, Leinonen M & Saikku P (2006) Inhibitory effect of heparan sulfate-like glycosaminoglycans on the infectivity of *Chlamydia pneumoniae* in HL cells varies between strains. Microbes Infect 8(3): 866–872

III Yan Y, Silvennoinen-Kassinen S, Törmäkangas L, Leinonen M & Saikku P (2008) Selective cyclooxygenase inhibitors prevent the growth of *Chlamydia pneumoniae* in HL cells. Int J Antimicrob Ag 32: 78–83

IV   Yan Y, Silvennoinen-Kassinen S, Leinonen M & Saikku P (2009) Rapamycin inhibits the development of *Chlamydia pneumoniae in vitro* in HL cells. Manuscript.

Some unpublished data are also presented.

Contents

1 Introduction

Chlamydia is an obligate intracellular Gram-negative bacterium which was originally thought to be a virus. *Chlamydia pneumoniae* is one of the species and is an important pathogen of acute and chronic respiratory tract infections in humans. *C. pneumoniae* infections are so common that antibodies are found in about 40–55% of the adults living in the northern hemisphere and in more than 60% in developing countries. However, more than 90% of infected people have only mild symptoms or even remain asymptomatic. Most importantly, *C. pneumoniae* causes persistent and recurrent respiratory tract infections which have been associated with chronic bronchitis, asthma and even with lung cancer (Blasi *et al.* 2002, Hahn *et al.* 1991, Kocazeybek 2003, Koyi *et al.* 2001, Laurila *et al.* 1997).

In addition to the acute or chronic, upper or lower respiratory tract infections, *C. pneumoniae* causes diseases in the cardiovascular system like myocarditis and endocarditis (Grayston 2000). An increasing number of studies have reported the association between the chronic *C. pneumoniae* infection and cardiovascular diseases (CVD) and atherosclerosis (Kalayoglu *et al.* 2002, Kuvin & Kimmelstiel 1999, Leinonen & Saikku 2002, Mendall *et al.* 1995, Saikku *et al.* 1988, Wong *et al.* 1999). Coronary heart disease (CHD) is the result of atherosclerosis of coronary arteries which supply blood and oxygen to the myocardium. While the atheromatous plaques accumulate on the inner walls of the coronary arteries, the arteries become hard and narrow. With the progress of atherosclerosis of the coronary arteries, the vulnerable plaques may progress to thrombosed plaques which rupture, causing an acute coronary syndrome including unstable angina, myocardial infarction, heart failure and sudden cardiac death.

Generally, the treatment for coronary artery disease, if atherosclerosis exists, includes administering drugs like aspirin and statins, eliminating risk factors as hypertension and diabetes, and performing coronary procedures depending on the symptoms and the development of the disease. Non-selective cyclooxygenase (COX) inhibitor aspirin and heparin-like antithrombin drugs can reduce the risk of blood clots, and some of calcium-channel blockers are commonly used to reduce the work load of heart by relaxing the arteries. Antiproliferation activity of rapamycin makes it useful in eluting stents for resisting in-stent restenosis (Kim *et al.* 2006).

The link between *C. pneumoniae* and atherosclerosis provides us with a possibility to prevent or treat CHD and other diseases caused by atherosclerosis with antibiotics; contrarily, the drugs used for cardiovascular disease may be

helpful in controlling the infection of *C. pneumoniae*, too. The cardiovascular drugs mentioned above have been reported to inhibit more or less a *C. trachomatis* infection, but only a few studies refer to their inhibitory effect on a *C. pneumoniae* infection (Tiran *et al.* 2002, Wuppermann *et al.* 2001). The effects of these drugs on *C. pneumoniae* might reveal possible new mechanisms for these drugs in the treatment of CVD.

2 Review of the literature

2.1 *Chlamydia*

2.1.1 Biological aspects of Chlamydia

Taxonomy

Chlamydia-like diseases were described as trachoma (sandy eyes) in China for over a hundred years, although the first isolation of the trachoma agent was achieved in China by Tang *et al.* in 1957 (Tang *et al.* 1957). Since then, more and more different chlamydial strains have been isolated and their classifications have had to be faced. Chlamydiae had been considered as viruses because they need a host cell to supply them with their metabolic energy; and thus, they can replicate only in an obligately intracellular environment, and they can also pass through bacterial filters. In 1963, Tamura and Higashi revealed that the main compositions of the chlamydial elementary bodies (EBs) are RNA and DNA by analyzing *C. psittaci* EB with radioactive ^{32}P label. The ribosomal RNA (rRNA) species present in the *C. psittaci* EB was the first indication that the chlamydiae may differ from viruses (Tamura & Higashi 1963). They were recognized as bacteria after Moulder contributed strong evidence for that in his studies (Moulder 1964). According to the original classification, there was only one order, *Chlamydiales*, which contained the one and only family, *Chlamydiaceae*, with only one genus, *Chlamydia* (Storz & Page 1971). Earlier the genus *Chlamydia* consisted of two species, *C. trachomatis* and *C. psittaci* (Page 1968). *C. trachomatis* was further subdivided into three biovars: trachoma which was divided into 14 serovars, A through K plus Ba, Da and Ia; lymphogranuloma venereum (LGV) divided into four serovars L1, L2, L2a and L3; and mouse (Moulder *et al.* 1984, Wang & Grayston 1991). Two decades later, two new species, *C. pneumoniae* (Grayston *et al.* 1989) and *C. pecorum* (Fukushi & Hirai 1992), were proposed. The original, or traditional, classification of *Chlamydia* is based on the morphological, physiological, biochemical, serological, developmental and genetic properties of the species. The genetic relationships are used to distinguish different species, for example, *C. trachomatis* biovar trachoma and LGV have almost 100% homological DNA, but when the mouse biovar is compared with biovar trachoma and LGV, the DNA relatedness is 30–60% (Kingsbury & Weiss 1968, Weiss *et al.* 1970). Recently, a new taxonomic

classification for the order *Chlamydiales* was proposed, which took into account recently identified *Chlamydia*-like obligately intracellular organisms. The order *Chlamydiales* was then reclassificated into four families, five genera and 12 species based on the phylogenetic analyses of the 16S and 23S rRNA genes (Everett *et al.* 1999, Rurangirwa *et al.* 1999). A comparison of the old and new classifications is given in Table 1. Although further speciation is needed, the new reclassification proposed by Everett *et al.* is not well accepted because it is based on minor sequence differences in 16S and 23S rRNA genes but not on the significant biological properties. Changing the name would create confusion to public health workers, funding agencies and so on (Schachter *et al.* 2001). Recently, Stephens *et al.* based classification on genomic sequences, deleted genus *Chlamydophila* and united all *Chlamydiaceae* in genus *Chlamydia* (Stephens *et al.* 2009). Chlamydiae are widely distributed in nature but only those existing in higher vertebrates have been studied. They prevalently exist in humans, mammals and birds but only a few of them cause diseases. Typical hosts of different species of *Chlamydia* are shown in Table 2.

Table 1. Comparison of chlamydial classifications.

	Order	Family	Genus	Species	biovar	serovar
Old	*Chlamydiales*	*Chlamydiaceae*	*Chlamydia*	*Chlamydia trachomatis*	trachoma	A, B, Ba, C, D, Da, E, F, G, H, I, Ia, J, K
					lymphogranuloma venereum (LGV)	L1, L2, L2a, L3
					muridarum	
				Chlamydia pneumoniae		
				Chlamydia psittaci		
				Chlamydia pecorum		
New	*Chlamydiales*	*Chlamydiaceae*	*Chlamydia*	*Chlamydia trachomatis*	Trachoma	
					lymphogranuloma venereum (LGV)	
				Chlamydia muridarum		
				Chlamydia suis		
			Chlamydophila	*Chlamydophila abortus*		
				Chlamydophila caviae		
				Chlamydophila felis		
				Chlamydophila pneumonia comb	TWAR; Koala Equine	
				Chlamydophila psittaci comb		
				Chlamydophila pecorum comb		
		Parachlamydiaceae	*Parachlamydia acanthamoebae*	*Parachlamydia acanthamoebae*		
		Simkaniaceae	*Simkania negevensis*	*Simkania negevensis*		
		Waddliaceae	*Waddlia chondrophila*	*Waddlia chondrophila*		

Table 2. Chlamydial species and typical hosts.

Classification	Species	Host
Old		
	Chlamydia trachomatis	human
	Chlamydia pecorum	cattle, sheep, goats, pigs, koalas etc.
	Chlamydia pneumoniae	human, koalas, horses
	Chlamydia psittaci	birds, sheep, cattle, cats
Proposed		
	Chlamydia trachomatis	human
	Chlamydia muridarum	mice, hamsters
	Chlamydia suis	swine
	Chlamydophila abortus	sheep, mammals
	Chlamydophila caviae	guinea pigs
	Chlamydophila felis	house cats
	Chlamydophila pecorum	cattle, sheep, goats, pigs, koalas etc.
	Chlamydophila pneumoniae	human, koalas, horses, lower vertebrates
	Chlamydophila psittaci	birds, sheep, cattle, cats
	Parachlamydia acanthamoebae	free-living amoebae*
	Simkania negevensis	acanthamoebae **
	Waddlia chondrophila	ruminants

* (Casson *et al.* 2006)

** (Kahane *et al.* 2001)

Structure

Chlamydiae are intracellular bacteria with their proliferation happening in the eukaryotic host cells. Depending on their developing cycle stage or phase, chlamydiae can be in either EB or RB form. The EB, elementary body, is the main form of extracellular chlamydiae and the RB, reticulate body, is the main form of intracellular chlamydiae.

Both EBs and RBs have a double layer cell membrane and the outer surface layer especially plays a crucial role in antigenicity and infectivity. This surface layer possesses a few important components. One main component is the major outer-membrane protein (MOMP), a 39.5 kDa outer membrane protein which maintains the rigidity of the chlamydial EB and is a serogroup-specific antigen of *C. trachomatis* (Caldwell *et al.* 1981, Hatch *et al.* 1981b). One of the important proposed functions of MOMP is to act as an adhesin to promote interactions with

host cells (Su *et al.* 1990). Structurally, MOMPs are heterogeneous between chlamydial species and considerably homologous among different *C. trachomatis* serotypes, but antigenically the MOMPs of different strains of *C. trachomatis* exhibit heterogeneity (Caldwell & Judd 1982, Caldwell & Schachter 1982). The structure and sequence of the *C. pneumoniae* MOMP gene is quite similar to those of other chlamydial species except for the differences of the sequence in the variable domains (Perez Melgosa *et al.* 1991). The MOMP of *C. pneumoniae* is a surface-exposed immunogenic protein, although it might be less immunogenic and antigenically complex than those of *C. trachomatis* and *C. psittaci* (Campbell *et al.* 1990, Wolf *et al.* 2001). Cysteine-rich outer-membrane proteins include outer membrane protein 2 (Omp2) of 60 to 62 kDa, Omp3 of 12.5 to 15.5 kDa and Omp4-5 of 97 to 99 kDa. Omp3 is not believed to be surface-exposed, but is anchored to the outer membrane by its lipid moiety with a hydrophilic peptide portion extending into the periplasm (Everett & Hatch 1995). Whether Omp2 is surface-exposed, there are conflicting opinions (Everett & Hatch 1995, Stephens *et al.* 2001, Ting *et al.* 1995). However, Omp4 and Omp5 have been shown on the surface of *C. pneumoniae* and the conformational epitopes of Omp4 seem to be dominant in infection *in vitro* (Knudsen *et al.* 1999). Additionally, the genes encoding a family of 21 polymorphic membrane proteins (Pmps) have been identified in *C. pneumoniae* genome and nine pmp genes in *C. trachomatis* genome (Kalman *et al.* 1999). The Pmps are not always stable. Several of them are variable in both molecular weight and expression within and between strains and are readily degraded (Grimwood *et al.* 2001). Although the Pmp 6, 8, 10, 11, 21 seem to be surface exposed (Vandahl *et al.* 2002) and Pmp 20, 21 are mediators of inflammatory activation of human endothelial cells *in vitro* (Niessner *et al.* 2003), the exact function of the Pmps are still enigmatic.

The genus-specific lipopolysaccharide (LPS) (Nurminen *et al.* 1983) is another important component located on the surface of both *C. pneumoniae* and *C. trachomatis* (Birkelund *et al.* 1989). It is an antigenic molecule: a general endotoxin of gram-negative bacteria (Kosma 1999). However the endotoxic activity of chlamydial LPS is much lower than that of the LPS of enterobacteria (Brade *et al.* 1987, Nurminen *et al.* 1983). Brade *et al.* showed that the main antigenic structure of this *Chlamydia*-specific LPS epitope is a trisaccharide of 3-deoxy-D-manno-octulosonic acid (Kdo) of the sequence Kdo*p*-(2→8)- Kdo*p*-(2→4)-Kdo (Brade *et al.* 1987).

Another group of proteins localizing on the outer membrane of *Chlamydia* and on other prokaryotic and eukaryotic cell membranes is heat shock protein (Hsp)

which is induced by a rise in environmental temperature or stress (Cooper & Ho 1983, Danilition *et al.* 1990, Ingolia *et al.* 1982, Key *et al.* 1981, Morrison *et al.* 1989, Wang *et al.* 1981). The chlamydial membrane contains three Hsp families: Hsp70, Hsp60 and Hsp10 (LaVerda *et al.* 1999, Peeling & Mabey 1999). As antigens, they play an important role in chlamydial infections. Hsp60 stimulates delayed-type hypersensitivity reaction in the macaque model of *C. trachomatis* salpingitis (Lichtenwalner *et al.* 2004); Hsp60 of *C. pneumoniae* is associated with asthma (Huittinen *et al.* 2001) and with CHD (Huittinen *et al.* 2003). Hsp10 may be associated with chronic genital tract infections of *C. trachomatis* (Betsou *et al.* 1999) and Hsp70 may be potentially involved in the attachment of chlamydiae to host cells thus related to adherence mechanisms (Raulston *et al.* 1993).

Macrophage infectivity potentiators (MIPs) are considered as virulence factors in intracellular pathogens such as *Legionella pneumophila* and *Trypanosoma cruzi*. *L. pneumophila* MIP (LpMIP) was the first member of this bacterial protein family identified as a virulence factor and shown to belong to the FK506-binding proteins (FKBPs, a group of immunophilins) family and to exhibit peptidyl-prolyl cis-trans isomerase (PPlase) activity (Cianciotto & Fields 1992, Fischer *et al.* 1992). The MIP protein has also been identified in *C. pneumoniae* (CpMIP) (Montigiani *et al.* 2002) and *C. psittaci* (Rockey *et al.* 1996), in which they seem to be surface-exposed immunodominant proteins found on both EBs and RBs. In addition, it has been demonstrated that the MIP homologue Cpn0661 of *C. pneumoniae* is a secreted effector protein providing the MIP protein with access into the host cell cytoplasm, and it can be demonstrated on the surface of inclusions from 20 to 72 hours post-infection (Herrmann *et al.* 2006).

Development cycle

Chlamydiae have a unique biphasic developmental cycle closely associated with infection. The chlamydial EB initiates the infection (or development cycle) by attaching, entering the host cell and forming inclusion; the EB differentiates into RB in the inclusion; the RB replicates by binary fission, then the RBs transform into new EBs and the new EBs are released from the inclusion and host cell. Chlamydial EBs and RBs are very different forms. The EB particles are small, about 0.2 to 0.3 μm in diameter and of high density. They are infectious but not able to replicate. Oppositely, the RBs are bigger, about 1 μm in diameter, of lower density, and they are able to replicate but are not infectious.

Attachment

The first step of chlamydial EB initiating the development cycle is to attach to the surface of host cells. Unfortunately, the mechanisms of the attachment of chlamydiae to host cells are still unclear. Chlamydiae may have adapted multiple means of attachment and entry. Studies in laboratories have revealed that many factors are associated with the attachment. Both extreme high and low temperatures can reduce the attachment. At 60 °C for 3 min, about 60–70% of attachment can be reduced (Hatch *et al.* 1981a); at 4 °C incubation temperature, it is halved but unaffected at 37 °C (Bose & Goswami 1986). Reversible electrostatic attractions and interactions between the surfaces of chlamydiae and host cells have been suggested to be involved in the attachment. Additionally, divalent cations (Ca^{2+}, Mg^{2+}, or Mn^{2+}) could enhance the attractions by neutralizing the negative charge on the surfaces of both chlamydiae and host. One or several different molecules, such as amino and carbohydrate groups on chlamydiae and glycoproteins on host cells, may contribute to these interactions (Hatch *et al.* 1981a). A few chlamydial proteins have been proposed to function as adhesion: MOMP is suggested to have a role as an adhesin (Su *et al.* 1990); 60 kDa cysteine-rich protein Omp2, which is only on chlamydial EBs, is believed to be involved in the attachment, entrance and avoidance of lysosomal fusion (Eissenberg & Wyrick 1981); and also Pmps are implicated to have a role in the process of chlamydial attachment (Longbottom *et al.* 1998). Glycosaminoglycans (GAGs) have been suggested as playing an important role in chlamydial attachment. When chlamydiae attach to eukaryotic cells, they may use heparan sulfate or heparin as receptors and bind the outer membrane proteins to them (Stephens *et al.* 2001). Therefore the exogenous heparan sulfate-like compounds could inhibit the infectivity of chlamydiae (Chen *et al.* 1996, Chen & Stephens 1997, Davis & Wyrick 1997, Stephens *et al.* 2000, Zhang & Stephens 1992). The heparan sulfate-like compounds were proposed to act as a trimolecular bridge between chlamydiae and the host cells (Zhang & Stephens 1992). However, differences exist between chlamydial species or biovars in their ability to utilize GAGs in attachment. *C. trachomatis* LGV may use the heparan sulfate-like GAGs on host cells as receptors, but *C. trachomatis* E may use other mechanisms; infectivity of *C. trachomatis* E on HeLa cells can be inhibited by chondroitin sulfate A, but not that of LGV (Taraktchoglou *et al.* 2001). An analysis of the outer membrane protein structure of trachoma and LGV revealed that the isoelectric points (pI) of Omp2 is 7.3 to 7.7 of trachoma, but > 8.5 of LGV; pIs of Omp3 are 5.4 of LGV but about 6.9 of trachoma (Batteiger *et al.* 1985). Such

structural differences may explain some of the biological differences between different species or strains. Relatively little is known about the situation of *C. pneumoniae*, and the possible differences between *C. pneumoniae* strains.

Internalization

As in the case of attachment, the mechanisms of the entry of attached chlamydial EBs into the host cells are uncertain. Chlamydial EBs have been proposed to enter nonphagocytic cells by pinocytotic or endocytotic mechanisms which are dependent on temperature, the functions of microfilaments and microtubules of the host cells and other conditions. Substances inhibiting the functions of microfilaments and microtubules can inhibit the endocytosis of attached chlamydiae (Ward & Murray 1984). Exogenous heparin can inhibit the infectivity of chlamydial trachoma biovar effectively but not so effectively as to inhibit the attachment (Chen & Stephens 1994). This implies that the receptor involved in the attachment binding the chlamydial heparan-sulfate-like molecule is also involved in the entry of the attached chlamydial EBs. For both *C. psittaci* and *C. trachomatis* E, Hodinka *et al.* found evididence of receptor-mediated endocytosis into clathrin-coated pits and of microfilament-dependent uptake into non-clathrin-coated vesicles (Hodinka *et al.* 1988). During studies, the mode of inoculation in experiments is a determining factor in the mechanisms of entry. With static incubation, pinocytotic mechanisms are more active; with centrifugation, endocytosis is more common (Prain & Pearce 1989). Additionally, a higher percentage of EBs is associated with lysosomes during uptake under static conditions implying that postinternalization events could be influenced by experimental protocols and mechanisms of entry. Therefore, the internalization of EBs into a host cell might use multiple mechanisms (Hackstadt 1999). *C. pneumoniae* may utilize the mannose 6-phosphate (M6P)/insulin-like growth factor 2 (IGF2) receptor for attachment and entry, whereas *C. trachomatis* may not use such a receptor (Puolakkainen *et al.* 2005). It seems that the mechanisms of chlamydial EBs entering host cells may vary between species or strains.

When entering the host cells, chlamydial EBs are wrapped in a membrane-bound vacuole to form inclusion, which can avoid phagolysosome formation and thus, digestion of the EBs can be prevented (Eissenberg & Wyrick 1981, Eissenberg *et al.* 1983). The chlamydial EBs tend to aggregate in the perinuclear region after their internalization to be inaccessible to antibodies (Majeed & Kihlstrom 1991). Their local aggregation is probably associated with

the fusion of inclusions (Ridderhof & Barnes 1989). The internalization of EBs escape from the fusion of lysosomes and initiation of the differentiation of EBs into RBs take place almost synchronously. During the processes when chlamydial EBs become enclosed endosomes, aggregate intracellularly, and form the chlamydial aggregates and inclusions, the intracellular Ca^{2+} plays a very important role. All these processes require a physiological concentration of intracellular ionized calcium ($[Ca^{2+}]_i$) (Majeed *et al.* 1993). The contact of chlamydiae with host cells can cause an intracellular calcium release and a disappearance of microvilli, which disrupts F-actin/β-tubulin cytoskeletal association with NF-κB/I κBα, inducing a NF-κB activation (Wissel *et al.* 2005). Once EBs begin a complex and lengthy reorganization into RBs, they lose their infectivity and increase in size and intensity of metabolic activity.

Proliferation

Proliferation of chlamydiae proceeds in their membrane-bound inclusion. The bacteria secrete proteins into the inclusion membrane and into the cytosol by a type III secretion mechanism and participate in giving the inclusion its unique properties (Dautry-Varsat *et al.* 2004). For multiplication to proceed, the EB wall must disappear and be replaced by the RB wall which has a different set of equally essential properties. Within 1 h of infection, the MOMP in the EB cell wall is reduced to monomers. By 8–12 h, reorganization of the EB into larger, non-infective RB via numerous morphologically intermediate stages is almost complete. As soon as they are mature, RBs start multiplication by binary fission and start to transform back into EBs (Collier 1990, Moulder 1991). When RBs are multiplying, the inclusion expands to occupy most of the cytoplasm of the host cell via the components obtained from both the host cell and the bacteria growing in the inclusion. By 20 h, the cysteine-rich proteins in the cell walls of the RBs have become linked by disulphide bonds, the content of RNA is 3–4 times greater than that of DNA, and 70S ribosomes have been formed. By this time, some small dense-centred particles are also visible and eventually greatly outnumber the intermediate forms. These are the new infectious EBs containing equal amounts of RNA and DNA within cell walls made rigid by disulphide bonding of cysteine-rich proteins. Divisions are not synchronous and for much of the growth cycle, RBs, EBs and intermediate forms can be seen together (Collier 1990, Moulder 1991). During the development of inclusion, RBs take nutrients and energy from the host cells (Wyrick 2000). Since the inclusion membrane is freely permeable to small

ions such as Ca^{2+}, inclusion obtains some metabolites such as amino acids and nucleosides, which have molecular masses of ~100–300 Da, using simple diffusion as a mechanism (Grieshaber *et al.* 2002). Chlamydiae may have the capacity to synthesize ATP (Iliffe-Lee & McClarty 1999, Read *et al.* 2000), but many intermediary metabolism genes are not expressed until mid-cycle although two ATP transporters are expressed early in development (Iliffe-Lee & McClarty 1999, Shaw *et al.* 2000). Thus, chlamydiae may scavenge ATP from cytoplasm of the host cell in the early stage of the developmental cycle.

Release

Finally, the developmental cycle ends with release of chlamydiae from host cells into the cytoplasm by exocytosis or host cell lysis. By means of transmission both within the infected host cell and to new host cells, the new infectious EBs attach to the new host cells to initiate a new developmental cycle in the new host cells. The release of the new infectious EBs happens 36–72 h after the infection, where variations are seen depending on species and strains. The inclusion membrane is disintegrated gradually with release of naked EBs into the host cytosol for later entry into uninfected host cells. Inclusions rotate rapidly, counterclockwise then clockwise, before a volcanic-like rupture to release chlamydial progeny within minutes (Wyrick 2000). There is no preferential release of EBs. RBs and intermediate forms left at the end of the developmental cycle, are set free along with the EBs. They are not infectious, but undoubtedly they are antigenic stimuli in intact hosts (Moulder 1991).

2.1.2 Pathogenetic aspects of Chlamydia

Chlamydia was considered to be only in human reservoirs for many years until isolations from other animals, birds, cattle, horses, koalas and so on, were obtained. As described above, different chlamydial species or biovars have different natural hosts. They infect different cells and cause different diseases. In fact, the infections caused by chlamydiae are complicated. Each strain may cause infections of various cell types and are associated with various diseases. *C. psittaci* does not cause psittacosis only among birds but also among humans (Center for Disease Control and Prevention 1998). *C. pneumoniae* is not only a pathogen of humans as considered before, but also exists in koalas, horses and a range of reptiles (snakes,

iguanas, chameleons) and amphibians (frogs, turtles) (Bodetti *et al.* 2002, Storey *et al.* 1993).

Generally, primary *C. trachomatis* infection occurs in genital organs after exposure to *C. trachomatis* inoculum, which is usually transmitted sexually. *C. trachomatis* biovar lymphogranuloma venereum (LGV) spreads from the site of primary infection by way of lymphatics, induces painful inguinal and femoral lymphadenopathy. *C. trachomatis* biovar trachoma spreads via secretions from person to person and causes ophthalmic disease, such as inclusion conjunctivitis and trachoma (Guze *et al.* 1981). When human respiratory tracts are exposed to large amount of *C. psittaci*, nasopharyngeal monocyte-macrophage can be invaded. By hematogenous dissemination, *C. psittaci* can spread to lungs and sites other than the pharynx. The hematogenous dissemination also distributes the organism to the liver, spleen, cardiac and skeletal muscle, and the central nervous system (Guze *et al.* 1981). Similar to *C. psittaci*, the infection of *C. pneumoniae* starts from respiratory tracts. Studies in animal models have revealed that *C. pneumoniae* can systemically disseminate in the body. The alveolar macrophages of lungs transport *C. pneumoniae* to peribronchiolar lymphatic tissue and peripheral blood and then *C. pneumoniae* enter the endothelial cells and smooth muscle cells (Rupp *et al.* 2005, Yamaguchi *et al.* 2006), and the spleen and aorta via dissemination by peripheral blood monocytes (Gieffers *et al.* 2004b). By this method, *C. pneumoniae* can disseminate in the body from one organ to another even into the central nervous system (Du *et al.* 2002). It can infect several cell types especially *in vitro*, such as epithelial cells, endothelial cells, vascular smooth muscle cells, peripheral blood mononuclear cells and monocyte-derived dendritic cells and macrophages (Airenne *et al.* 1999, Gaydos *et al.* 1996, Wittkop *et al.* 2006), lymphocytes (Haranaga *et al.* 2001), brain cells (Boelen *et al.* 2007), cardiomyocytes (Spagnoli *et al.* 2007) and adipocytes (Bouwman *et al.* 2008).

Chlamydial infection is characterized by repeated and persistent infection. The tissue damage and scarring are the outcomes of the reinfection. The mechanisms by which the reinfection causes such inflammatory results are not well known. However, it is believed that chlamydial Hsps play an important role in the pathogenesis of disease. Within the Hsp families, Hsp60 seems to be the key antigen in chronic chlamydial infections. The scarring tissue damage in trachoma is associated with Hsp60 targeted immune responses (Peeling & Mabey 1999). Chlamydial Hsp60 localizes in human atheromatous tissue (Kol *et al.* 1998) and is also associated with the development of atherosclerosis (Xu *et al.* 1993). A persistent infection model affected by interferon gamma (IFN-γ) revealed that

chlamydial Hsp60 kept its production near a normal level while the synthesis of the MOMPs, Omps, lipopolysaccharides and other structure proteins were greatly reduced (Beatty *et al.* 1993, Beatty *et al.* 1994).

It has also been demonstrated that chlamydial infection causes a 10-fold increase of the expression of the cyclooxygenase-2 (Cox-2) mRNA in the peripheral blood monocytes (Rupp *et al.* 2004). Cox catalyses the biosynthesis of prostaglandins (PGs) from arachidonic acid such as prostacyclin (PGI_2), thromboxane A_2 (TXA_2) and prostaglandin E_2 (PGE_2). The enhanced expression of Cox-2 and prostaglandin E_2 (PGE_2) in monocytic cells is associated with increased plaque instability in human atheromas (Cipollone *et al.* 2001).

One of chlamydia-induced responses in the immune system is the expression of cytokines by either infecting host cells directly or by interaction with the immune system cells (Fitzpatrick *et al.* 1991). After infection with *C. trachomatis* and *C. psittaci*, cervical and colonic epithelial cells upregulate mRNA expression and secretion of the proinflammatory cytokines: interleukin 8 (IL-8), growth-related oncogene protein-α (GRO-α), granulocyte-macrophage colony-stimulating factor (GM-CSF), and IL-6. Epithelial cell lines and primary endocervical epithelial cells release IL-1α, which may amplify the inflammatory response by stimulating additional cytokine production by noninfected neighboring cells since the increased secretion of the proinflammatory cytokines could be inhibited by anti-IL-1α (Rasmussen *et al.* 1997). Much less is known about *C. pneumoniae* than *C. trachomatis* immunity. Nevertheless, it is suggested that they have a similar immunobiologic paradigm. The monocytes infected with *C. pneumoniae* increase the expression of CD14 and secrete tumor necrosis factor alpha (TNF-α), IL-1β and IL-6 (Heinemann *et al.* 1996). During infection of *C. pneumoniae*, antibody responses may be immunopathological under certain conditions. In the serum of patients with chronic or acute coronary heart diseases, circulating immune complexes contain *C. pneumoniae* antigen and antibodies (Leinonen *et al.* 1990, Linnanmaki *et al.* 1993).

A large number of human infectious diseases are attributed to the chlamydial infection (Schachter & Dawson 1978). Psittacosis is the typical disease caused by *C. psittaci* (Center for Disease Control and Prevention 1998). The diseases caused by *C. trachomatis* are primarily trachoma which develops from a mucopurulent conjunctivitis to a follicular keratoconjunctivitis and leads to blindness (Dawson 1975), lymphogranuloma venereum manifesting acute inguinal lymphadenitis and complicating with later rectal, urethral and genital lymphedema, and genital infections which include nongonococcal urethritis in male, cervicitis, urethritis,

salpingitis, postpartum endometritis and so on in females (Schachter & Dawson 1978). Here, the reviewed literature is mainly related to the infections by *C. pneumoniae* species.

Acute C. pneumoniae infections

It is believed that *C. pneumoniae* transmits from person to person via an airborne route. Although the efficiency of this transmission is very low, host-to-host transmission is a relatively rapid process which occurs via sneezing and coughing. *C. pneumoniae* can survive in aerosol at room temperature and high relative humidity supports the possibility of a direct transmission from person to person (Theunissen *et al.* 1993). The *C. pneumoniae* infection may also be acquired by the transmission from asymptomatic carriers. The incubation period is about 1 to 3 weeks and, thus, symptoms may appear much later after exposure than with most respiratory infections caused by other pathogens. *C. pneumoniae* infections are often mild or even asymptomatic. Symptomatic acute infections manifest most commonly as bronchitis and pneumonia, which are usually mild but may also be severe especially among the elderly. Typically, the acute infection of *C. pneumoniae* has a biphasic illness. Before the appearance of typical bronchitis and pneumonia symptoms, a subacute onset of pharyngitis, sometimes with hoarseness, and with a persistent non-productive cough is common. Usually, there is no fever, or if present, the fever is low grade. Sinusitis may occur in connection with *C. pneumoniae* infection (Blasi *et al.* 1994a, Blasi *et al.* 1994b, Kuo *et al.* 1995). An acute *C. pneumoniae* infection has been suggested to lead to acute severe asthma exacerbation (Cosentini *et al.* 2008, Lieberman *et al.* 2003). Other than an upper and lower respiratory infection, an acute *C. pneumoniae* infection can be even associated with neurological disease (Koskiniemi *et al.* 1996) e.g. acute disseminated encephalomyelitis (ADEM) although the disease itself is rare (Heick & Skriver 2000). Recent animal studies also revealed the acute infection of *C. pneumoniae* can cause a change in the trace element balance in infected organs (Edvinsson *et al.* 2008a, Edvinsson *et al.* 2008b) which might induce sequential symptoms or diseases.

Since there are no specific or unique symptoms in the respiratory infection caused by *C. pneumoniae*, it is difficult to distinguish it from infections by other aetiological agents. Additionally, it is quite common that *C. pneumoniae* is mixed with other causative agents such as *Streptococcus pneumoniae* and *Mycoplasma pneumoniae* (Kauppinen *et al.* 1995). In laboratory diagnosis, serological tests to

measure IgM, IgA or IgG antibodies by EIA, MIF and CF are reasonable methods for the acute *C. pneumoniae* infection (Ekman *et al.* 1993, Kuo *et al.* 1995, Miyashita *et al.* 2008a). To diagnose primary infection, an immunochromatographic test for the detection of *C. pneumoniae*-specific IgM antibodies has been reported recently by Miyashita, and with this test the diagnosis of *C. pneumoniae* pneumonia can be made quickly (Miyashita *et al.* 2008b).

Persistent C. pneumoniae infection

If the acute infection has not been eradicated, *C. pneumoniae* can live in host persistently, and elicit recurrent and persistent infection leading to chronic damage of the host's organs. The development of a persistent infection can be due to delayed or inadequate antimicrobial therapy (de Kruif *et al.* 2005, Gieffers *et al.* 2004a), since acute infection is often mild or even asymptomatic.

In persistent infection, the host's response is not able to eliminate the microbe and the continued presence and replication of a microbe in the host are accompanied with a variable degree of, but continued, damage to the host. Progressive damage from this state may lead to disease and death (Casadevall & Pirofski 2000). Experiments *in vitro* have shown that persistent infection can be induced by the presence of certain antibiotics, IFN-γ and TNF-α, etc., especially in subinhibitory concentrations (Beatty *et al.* 1994, Gieffers *et al.* 2004a, Pantoja *et al.* 2000, Pantoja *et al.* 2001, Summersgill *et al.* 1995), or insufficient supply of nutrients such as iron (Wehrl *et al.* 2004). *In vitro*, the persistent state is characterized by a loss of infectivity, since chlamydial EBs do not differentiate into normal RBs, but form large pleomorphic noninfectious aberrant bodies (ABs). The ABs do not redifferentiate back into infectious EBs as in the normal life cycle. Thus, *C. pneumoniae* in a persistent state is metabolically active but non-cultivable and has differences in gene and protein expression patterns compared acute infection (Beatty *et al.* 1994, Beatty *et al.* 1995, Belland *et al.* 2003, Byrne *et al.* 2001, Gerard *et al.* 2002, Mathews *et al.* 2001). Persistent *C. pneumoniae* infections have been studied in animal experiments but the mechanisms are still unclear. The presence of a chronic *C. pneumoniae* infection can possibly be diagnosed by the continuous presence of high IgG or IgA titers in sera or by the presence of antigen or DNA in the infected tissues but not by *Chlamydia* culture (Kuo *et al.* 1993, Laurila *et al.* 1997).

In fact, the diagnosis of the chronic *C. pneumoniae* infections is problematic. A "hit-and-run" situation is the most difficult possibility in which *C. pneumoniae*

itself has escaped after inducing an abnormal immunological reaction, a self-supporting process causing the progression of the disease (Saikku 1999). Like in acute infections, the diagnostic methods of the persistent chronic *C. pneumoniae* infection are mainly based on the use of serological test and nucleic acid detection from infected tissues. The lack of typical clinical manifestations and the culture-negative stage do not mean the microbe persistently existing in host body is an innocent bystander. The process by which *C. pneumoniae* causes damage to the host may be much longer than in common infections. It, for instance, takes tens of years for a *C. trachomatis* infection to develop trichiasis and entropion, the blinding lesions of trachoma (Schachter 2004). Numerous studies have shown that many chronic respiratory infections are associated with an acute or chronic *C. pneumoniae* infection. *C. pneumoniae* may be an aetiological agent for chronic pharyngitis (Falck *et al.* 1997) and is associated with a higher rate of exacerbations of chronic bronchitis (Blasi *et al.* 2002). Several studies have reported the association between *C. pneumoniae* and asthma and chronic obstructive pulmonary disease (COPD) (Biscione *et al.* 2004, Branden *et al.* 2005, Hahn *et al.* 1991, Hahn 1999, Von *et al.* 2002). A chronic *C. pneumoniae* infection may also be a risk factor in lung cancer (Kocazeybek 2003, Koyi *et al.* 2001, Laurila *et al.* 1997, Littman *et al.* 2005). In addition to respiratory infections, the persistent *C. pneumoniae* infection has been suggested to have a causative role in neurological diseases such as in Alzheimer's disease, where *C. pneumoniae* is found in high frequency in glial cells within the brains of the patients (Balin & Appelt 2001) with meningitis (Sundelof *et al.* 1993), encephalitis (Airas *et al.* 2001) and multiple sclerosis (MS) (Sriram *et al.* 1999). The persistent existence of *C. pneumoniae* in synovium is probably associated with chronic arthritis (Villareal *et al.* 2002). In the persistent *C. pneumoniae* infection of the non-respiratory system, the microbe needs to be transmitted from the lungs to target sites via the bloodstream. Here, alveolar macrophages act as vehicles to disseminate *C. pneumoniae* to the vascular wall and then to other tissues (Gieffers *et al.* 2004b). The large number of studies have associated the persistent *C. pneumoniae* infection to cardiovascular diseases: coronary artery disease (Goyal *et al.* 2007), severe essential hypertension (Cook *et al.* 1998), coronary atheromas (Borel *et al.* 2008), acute ischaemic stroke (Madre *et al.* 2002, Wimmer *et al.* 1996), myocardial infarction (Spagnoli *et al.* 2007) and cerebrovascular accidents (Bucurescu & Stieritz 2003).

In 1988, it was first described that chronic *C. pneumoniae* infection is probably associated with chronic coronary heart disease when seroconversion against an epitope of chlamydial LPS was demonstrated during acute myocardial infarction

(Saikku *et al.* 1988). Later serological studies also showed that the elevated prevalences of the specific antibodies IgA and IgG are significantly associated with atherosclerosis and increased risk for cardiovascular diseases in such patients (Halfon *et al.* 2006, Tasaki *et al.* 2003, Wolf *et al.* 2003). Increased *C. pneumoniae* IgA antibody titer is associated independently with elevated concentrations of fibrinogen which is related to a poor outcome in unstable coronary artery disease (Toss *et al.* 1998). Wolf *et al.* reported that elevated IgA titers were found in 67% of symptomatic atherosclerotic patients when 29% were from the patients without atherosclerotic symptoms, and 57 of 151 symptomatic atherosclerotic patients died after three years but only 20 of 116 patients without such symptoms died (Wolf *et al.* 2003). Nevertheless the serological studies have some limitations because they usually do not indicate when the infection occurs or its spatial relationship to start of atherosclerosis (Shor 2007). Detection of *C. pneumoniae* in atherosclerotic plaques, or atheromas, with PCR, electron microscope and immunocytochemistry, etc., supplied the evidences that *C. pneumoniae* is present in the atherosclerotic plaques of coronary artery, carotid and aorta, and thus confirmed the association between this microbe and atherosclerosis (Campbell *et al.* 1995, Juvonen *et al.* 1997a, Juvonen *et al.* 1997b, Kuo *et al.* 1993, Kuo *et al.* 1993, Shor *et al.* 1992). Although *Helicobacter pylori* has also been suspected of being associated with cardiovascular disease, only *C. pneumoniae* DNA, but not *H. pylori* DNA, was detected in atherosclerosis plaques (Dore *et al.* 2003). Animal models also linked the infection of *C. pneumoniae* to atherosclerosis. In a rat model, a chronic *C. pneumoniae* infection may cause an atherosclerotic process in the aorta (Aziz 2006). The results in rabbit models (Fong *et al.* 1997, Laitinen *et al.* 1997, Muhlestein *et al.* 1998) showed *C. pneumoniae* increased the atheromatous plaque formation significantly, and this was prevented by early antibiotic treatment (Fong *et al.* 1997). The elevation of several risk factors for atherosclerosis, CRP, leukocytes and some cytokines, induced by a chronic *C. pneumoniae* infection may be the mechanisms by which *C. pneumoniae* infection is associated with atherosclerosis (Leinonen 2000). The presence of some factors, immune complexes, antigenic mimicry of Omp2, endotoxin and Hsp 60 of *C. pneumoniae*, also indicate the association between the pathogen and disease (Saikku 2000). In the past two decades, a huge amount of studies evaluated the role of *C. pneumoniae* in atherosclerosis. Except for the abovementioned evidence which confirmed the association, there are also studies giving their negative findings in animal experiments (Caligiuri *et al.* 2001) and antibiotic treatment trial on mice in which azithromycin treatment could not reduce the atherosclerosis exacerbated by *C.*

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pneumoniae infection (Rothstein *et al.* 2001). As Watson and Alp concluded, based on all present evidence, *C. pneumoniae* may be neither alone sufficient nor be necessary to cause atherosclerosis or its clinical consequences in humans (Watson & Alp 2008).

2.1.3 Treatment of chlamydial infection

Currently, the treatment used routinely for a chlamydial infection is antibiotic therapy. As gram-negative bacteria, chlamydiae are susceptible to antibiotics with wide antimicrobial spectrum activity. Tetracycline and its congeners exhibit antimicrobial activity against a broad range of gram-positive and gram-negative bacteria, chlamydiae, mycoplasmas rickettsiae, *Toxoplasma gondii* and protozoan parasites. Therefore, they are also the agents of choice for treating chlamydial infections (Francke & Neu 1987, Roberts 2003). Other than tetracycline, other antibiotics have been shown to be as good or have an even better efficacy in the treatment of chlamydial infection: doxycycline (Su *et al.* 1999), macrolides: erythromycin (Hunter & Sommerville 1984, Patamasucon *et al.* 1982); penicillins: pivampicillin (Moller *et al.* 1985), amoxicillin-clavulanic acid (Beale & Upshon 1994); fluoroquinolones: fleroxacin, ofloxacin (Ronald & Peeling 1991, Schachter & Moncada 1989); azithromycin (Tuffrey *et al.* 1991), rifalazil (Suchland *et al.* 2006), rifampin (Carter *et al.* 2004), roxithromycin (Stenberg & Mardh 1993) and so on. In practice, the treatment of chlamydial infections is quite complicated. Since chlamydiae are obligate intracellular gram-negative bacteria, their treatments require antibiotic drugs to pass the membranes of both of the host cell and the organism. Different antibiotics may have different effectory mechanisms. The antibiotics may give inconsistent results between susceptibility tests *in vitro* and results of therapeutic trials. A combination of antibiotics can increase their effect (Carter *et al.* 2004, Ridgway & Oriel 1984). Most of the effective means of treatment are for acute chlamydial infection, but the real difficult cases are chronic ones. Although antibiotics can eradicate chlamydiae effectively, they may also inhibit the development of protective immunity thereby reinfection commonly occurs (Su *et al.* 1999). Antibiotics themselves may also cause persistence infection (Gieffers *et al.* 2004a).

In addition to the antibiotics, small inhibitory molecules of *Yersinia* type III secretion system, *N'*-(3,5-dibromo-2-hydroxybenzylidene)-4-nitrobenzohydrazide and the purinergic receptor P2X$_7$R are recently reported to be able to disrupt the progression of the chlamydial developmental cycle or directly inhibit the

chlamydial infection as well as antibiotics (Darville *et al.* 2007, Wolf *et al.* 2006). For *C. pneumoniae*, Alvesalo *et al.* have screened natural flavonoids and other natural and structurally similar synthetic compounds, for their antichlamydial effect (Alvesalo *et al.* 2006). Table 3 lists the most effective ones.

Table 3. Most effective antichlamydial phenolic compounds derived from plants or synthesized (modified from Alvesalo et al., 2006) (Alvesalo *et al.* 2006).

Type of compound	Compound	Inhibition (%)
Flavones	Acacetin	100
	Apigenin	100
	Flavone	90
	Luteolin	100
Flavonols	Morin	100
	Myricetin	100
	Rhammetin	100
	Quercetin	90
Synthetic flavonoids	α-Naphtoflavone	92
	2'-Methoxy-α-naphtoflavone	100
	Rotenone	100
Natural coumarins	Methoxy psoralen	100
	Scopoletin	96
	Xanthotoxin	94
	Umbelliferone	75
Synthetic coumarins	7-Diethylamino-3-thenoylcoumarin	100
	Coumarin 106	100
Catechins	(+)-Catechin	76
	(-)-Epicatechin	75
Phenolic acid	Caffeic acid	78
Gallates	Dodecyl gallate	100
	Methyl gallate	100
	Octyl gallate	100
	Propyl gallate	100
	(-)-Epicatechin gallate	85

2.2 Cardiovascular diseases and atherosclerosis

As defined by the World Health Organization (WHO), cardiovascular diseases (CVD) are diseases caused by disorders of the heart and blood vessels, and include coronary heart disease (heart attacks), cerebrovascular disease (cerebrovascular disease), raised blood pressure (hypertension), peripheral artery disease, rheumatic heart disease, congenital heart disease and heart failure. Important clinical manifestations of CVD are acute coronary syndromes (ACS), stroke, renal failure and occlusive peripheral vascular disease (PVD).

The major pathological process of these cardiovascular diseases in different vascular beds is atherosclerosis, a chronic inflammatory disease of systemic arteries (Ross 1999). It is initiated when the endothelium of an artery is activated by multiple risk factors such as smoking, hypercholesterolaemia, hyperglycaemia, diabetes, hypertension and circulating immune complexes. The hemodynamic strain and the accumulation of low-density lipoproteins (LDL) may initiate an inflammatory process in the arterial wall (Dai *et al.* 2004, Skalen *et al.* 2002). Before the lesions of atherosclerosis are formed, the permeability of endothelial intima is increased to lipoproteins, prostacyclin, platelet-derived growth factor, angiotensin II and endothelin. Leukocyte adhesion molecules, such as L-selectin, integrins, and platelet-endothelial-cell adhesion molecule 1, and endothelial adhesion molecules, as E-selectin, P-selectin, intercellular adhesion molecule 1, and vascular-cell adhesion molecule 1, are up-regulated. Leukocytes, monocytes and T cells, then migrate into the artery wall. In patients with hypercholesterolemia, excess LDL infiltrates the artery and accumulates in the intima. Fatty streaks initially consist of lipid-laden monocytes and macrophages (foam cells) together with T lymphocytes. Later they are joined by various numbers of smooth-muscle cells (Ross 1999). LDL inhibit endothelial function by down-regulation of endothelial NOS expression and decrease receptor-mediated nitric oxide (NO) release (Liao 1994, Liao *et al.* 1995). Lack of NO in atherosclerotic vessels could impair vascular relaxation and platelet aggregation, increase vascular smooth muscle proliferation, and enhance leukocyte adhesion to the endothelium (Garg & Hassid 1989, Kubes *et al.* 1991, Radomski *et al.* 1990, Tanner *et al.* 1991). Monocytes pass through the endothelium of artery into the wall of the artery and transform into macrophages, then collect oxidized cholesterol and develop into foam cells. Connective and elastic tissues, lipid, cholesterol, cell debris, calcium, fibrin and other substances also accumulate there building up plaque in the inner lining of an artery. This plaque is called atheroma or atherosclerotic plaque which

thickens the arterial wall and bulges into the lumen of the artery. The atherosclerotic plaques may narrow or block an artery, reducing or stopping blood flow (Fig. 1). A stable plaque may cause a local stable angina.
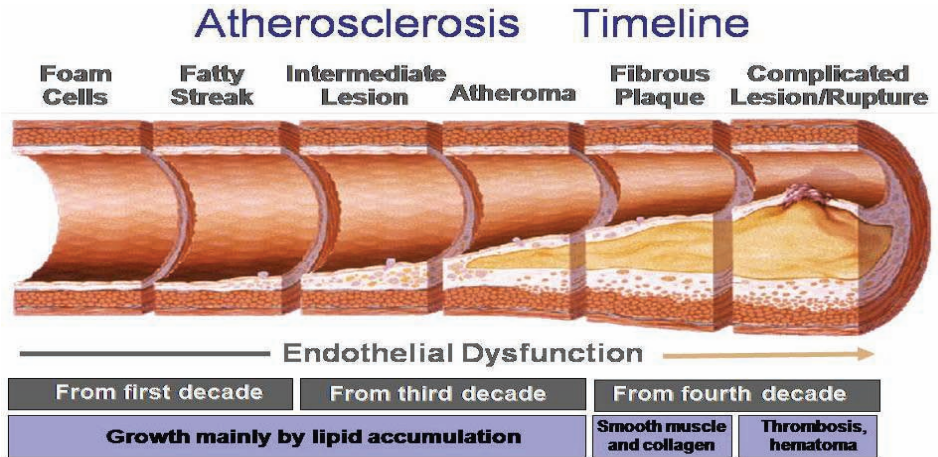
Fig. 1. Progression of atherosclerosis (adapted from Stary HC *et al.* (Stary *et al.* 1995)).

Within the sites of atherosclerotic plaque, activated macrophages, T cells and mast cells produce numerous inflammatory molecules, such as inflammatory cytokines, proteases, coagulation factors, radicals and vasoactive molecules, and proteolytic enzymes. They inhibit the formation of stable fibrous caps, activate cells in the core, and attack collagen in the cap; destabilize the stable plaque into a vulnerable, unstable structure with a risk of plaque rupture and exposure of the thrombogenic lipid core to blood; initiate thrombus formation, then induce the activation and rupture of the 'unstable plaque', thrombosis and ischemia – an acute coronary syndrome (Hansson 2005).

The aetiology of atherosclerosis is complicated and there are many risk factors associated with the development of atherosclerotic plaque. In addition to important unmodifiable risk factors like age, sex and genetics, the most important and modifiable risk factors are smoking, hypercholesterolemia, diabetes or hypertension, abdominal obesity, low consumption of fruits and vegetables, low physical activity, high alcohol consumption, and psychosocial stressors (Yusuf *et al*. 2004). Among them, smoking is strongly associated with atherosclerosis and cardiovascular diseases (Cerami *et al.* 1997, Howard *et al.* 1998, Roberts *et al.* 1996). The relationship between infection, inflammation, and atherosclerosis has

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been intensively investigated during the past decade. Sero-epidemiological and histological studies support the association between atherosclerosis and infection with *C. pneumoniae* and cytomegalovirus (CMV) (Apfalter *et al.* 2000, Campbell *et al.* 1995, Grayston 2000, Maass *et al.* 2000, Melnick *et al.* 1993, Ramirez 1996, Saikku *et al.* 1988). These agents are able to initiate and accelerate atherosclerosis in animal models. If they cause persistent infection in the vessel wall, they can directly promote a proinflammatory, procoagulant, and proatherogenic environment (Leinonen & Saikku 2002). *C. pneumoniae* can induce macrophages to take native unmodified LDL, which is normally not important in the formation of foam cells, to become foam cells. Chlamydial LPS and cHsp60 can induce oxidation of LDL within the neointima, providing a mechanism by which *C. pneumoniae* could enhance foam cell formation (Watson & Alp 2008). An endothelial response to chronic infection of CMV and *C. pneumoniae* may be enhanced by intracellular iron (Kartikasari *et al.* 2006). Although evidence shows the pathogenic role for *C. pneumoniae* in atherosclerosis, for a multifactorial disease it is difficult to ascribe causality to this pathogen (Watson & Alp 2008). The causality of atherogenesis from the infection of *C. pneumoniae* and CMV still needs more research.

2.3 Drugs for the treatment of cardiovascular diseases

Cardiovascular diseases are caused by multiple factors. Symptoms appear mainly when a blood vessel, especially a coronary artery, becomes stenotic or is thrombotic due to a stable atherosclerosis plaque or rupture of an unstable plaque. The clinical treatment for cardiovascular diseases includes administering aspirin and a coronary procedure. For a patient with acute myocardial infarction, antithrombotic drugs are needed. To prevent the occurrence of cardiovascular disease, a change in lifestyle is necessary. Table 4 lists some of the most commonly used drugs during the treatment of cardiovascular diseases. Additionally, COX inhibitors and rapamycin are also used in the treatment.

Table 4. Most commonly used drugs in treating cardiovascular diseases (Illingworth 1988a, Illingworth 1988b, Manickavasagam *et al.* 2009, Ocava *et al.* 2006, Strony *et al.* 2008).

Clinical use of drug	Type of drug	Example drug
Acute coronary syndromes, stable angina and stroke	Antiplatelet drugs	aspirin clopidogrel
	Statins	lovastatin simvastatin
	β-blockers	bisoprolol metoprolol
	Angiotensin-converting enzyme (ACE) inhibitors	perindopril enalapril ramipril
	Angiotensin II receptor blockers (AT blockers)	valsartan losartan olmesartan
	Anticoagulants	enoxaparin heparin
	Glycoprotein IIB/IIIA Inhibitors	tirofiban abciximab eptifibatide
	Antithrombotic drugs	tPA (tissue plasminogen activator)
	Vasodilators	isosorbide dinitrate
	Diuretics	amiloride chlorothiazide
	Calcium channel blockers	verapamil amlodipine

2.3.1 Heparins

Heparin is a glycosaminoglycan (GAG), an alternating copolymer of an uronic acid and an amino sugar. It is composed of heterogeneous chains with varying molecular weights and anticoagulation activity. Its linear polysaccharides activate antithrombin III (ATIII) dramatically enhancing its ability to inhibit thrombin and factor Xa. Heparin chains can be cleaved by a method known as heparin depolymerization because of the uneven distribution of different sequences along the heparin chains. This cleavage produces low molecular weight heparins (LMWHs) with different lengths of heparin chains, molecular weights and anticoagulant activity for study and clinical use. Both unfractionated heparin and LMWHs are used as part of standard treatment of ACS (Antman *et al.* 2004, Le Nguyen & Spencer 2001).

GAGs are bound to the protein core in proteoglycans, which are present in the extracellular matrix and membranes of virtually all animal cells. Several pathogenic microbes, both bacteria and viruses, have learned to exploit them as receptors for their attachment to their target cells (Rostand & Esko 1997). The antiviral activities of heparin and its derivatives have been found for many years. It was found that heparin had an extremely potent inhibitory effect on the infectivity of herpes simplex virus (HSV) *in vitro* (Nahmias & Kibrick 1964, Vaheri 1964) early in 1964 which was confirmed with different strains of HSV, different tissue culture and different media. Heparin was also found to have inhibitory effect on the human papillomavirus (Villanueva *et al.* 2006) and on human immunodeficiency virus (HIV) infection by altering HIV envelope reduction by protein disulfide-isomerase in addition to the direct effect on the interaction of the envelope with cell-surface components (Barbouche *et al.* 2005). Clinical and experimental evidence suggest that heparin also possesses anti-cancer activities. Heparin seems to inhibit the metastasis formation of cancer by inhibiting selectin-mediated interactions of tumor cells with leukocytes, platelets, and endothelial cells, which are likely to mediate the initial steps of hematogenous metastasis, rather than to affect the growth of primary tumors (Laubli & Borsig 2009, Niers *et al.* 2007). LMWHs offer a number of potential advantages over unfractionated heparin in the management of ACS. They have a higher antithrombotic activity. By inhibiting a small amount of factor Xa, they prevent the formation of considerably larger amounts of thrombin. In postsurgical and medical prophylaxis of acute deep-vein thrombosis, LMWHs showed the benefits (Breddin *et al.* 2001, Saltissi *et al.* 1999, Verheugt & Chesebro 2001). Potential advantages

of LMWHs also include a high bioavailability after subcutaneous administration; more predictable anticoagulant effect, which avoids the need for therapeutic monitoring; and decreased sensitivity to platelet factor 4 (Antman & Handin 1998). In certain clinical trials LMWHs improved survival of cancer patients with deep venous thrombosis, which may be partially dependent and partially independent on its anti-coagulant activities (Mousa & Petersen 2009).

As described in chapter Development cycle, chlamydiae invade eukaryotic cells by binding heparan sulfate or heparin with their outer membrane proteins (Stephens *et al.* 2001). Heparan sulfate-like GAGs are used by *C. trachomatis* as receptors when attaching to its host cells; and the exogenous heparan sulfate-like GAGs have been shown to inhibit the infectivity of both *C. trachomatis* and *C. pneumoniae* (Beswick *et al.* 2003, Kuo & Grayston 1976, Wuppermann *et al.* 2001). The LMWH Clexane (enoxaparin) has also shown the strongest inhibitory effect on the infectivity of *C. trachomatis* (Taraktchoglou *et al.* 2001).

2.3.2 Cyclo-oxygenase inhibitors

Cyclo-oxygenase (prostaglandin endoperoxide synthase) (COX) is the enzyme which catalyses the biosynthesis of prostaglandins (PGs) from arachidonic acid, regulating the synthesis of eicosanoids, such as prostacyclin (PGI_2), thromboxane A_2 (TXA_2) and prostaglandin E_2 (PGE_2). Prostaglandins play important roles in inflammation and the maintenance of normal physiological function of several organ systems, and in the pathophysiological mechanism of action of platelets and endothelial cells in the cardiovascular system. Increased biosynthesis of PGI2 and TXA2 is associated with atherosclerosis (FitzGerald *et al.* 1984). There are two isoforms of the COX enzyme, COX-1 and COX-2. They are encoded by distinct genes (Hla & Neilson 1992, Vane *et al.* 1998, Xie *et al.* 1991) and act in different ways. Normal physiological functions are mediated by COX-1 which is constitutively expressed in most tissues, while the inflammatory response is mediated by COX-2 which is expressed when induced by inflammation (Burleigh *et al.* 2002, O'Neill & Ford-Hutchinson 1993). Both of them have been found to be expressed or overexpressed in human atherosclerotic lesions, and especially COX-2 is expressed only in atheromatous but not in normal arteries (Baker *et al.* 1999, Belton *et al.* 2000, Burleigh *et al.* 2002, McGeer *et al.* 2002, Schonbeck *et al.* 1999, Stemme *et al.* 2000). Activated platelets show a relatively large amount of COX-1, whereas endothelial cells have the gene for COX-2, the expression of which follows cell activation. In the atherosclerosis lesion, prostaglandin synthesis

is mainly mediated by the inducible COX-2 expressed in macrophages/foam cells, smooth muscle cells and endothelial cells. These implicate that the inhibition of COX-2 may be beneficial in atherosclerosis (Burleigh *et al.* 2002).

Both of COX-1 and COX-2 can be inhibited by non-steroidal anti-inflammatory drugs (NSAIDs) such as aspirin and ibuprofen (Marnett *et al.* 1999). The COX active site in prostaglandin H_2 synthase is at the end of a long, hydrophobic channel, which runs from the protein's membrane-binding surface deep into the interior of the molecule, to the site where NSAID is binding (Picot *et al.* 1994). By acetylating a serine residue positioned in the channel (Ser[529] of COX-1 and Ser[516] of COX-2), unique among NSAIDs, aspirin covalently modifies COX thus irreversibly inactivating it and preventing the generation of prostaglandins and thromboxane A_2 (Loll *et al.* 1995). In fact, this acetylation has no direct effect on the COX activity but it prevents the access of the substrate to the catalytic site of the enzyme (Loll *et al.* 1995). With this function, aspirin displays its antiplatelet, antithrombotic effect on cardiovascular disease (Antman *et al.* 2004). Aspirin improves endothelial dysfunction and enhances the vasodilation in response to acetylcholine in patients with atherosclerosis (Husain *et al.* 1998). The four main reasons for using aspirin are that it can significantly decrease the expression of COX-2, the maximum thickness of aorta plaque, the artery stenosis and the proportion occupied by atheroma in the intima; thus it delays the development of atherosclerosis significantly (Guo *et al.* 2006). Aspirin also offers protection against stroke and thrombosis, Alzheimer's disease and cancer (Giovannucci *et al.* 1995, Pasinetti 1998). Additionally, aspirin has recently been shown to inhibit the growth of *C. pneumoniae* (Tiran *et al.* 2002, Yoneda *et al.* 2003).

NSAIDs, such as aspirin, ibuprofen and diclofenac, inhibit the constitutively expressed COX-1 in the gastric mucosa to produce gastric damage, and to cause adverse effects in the gastrointestinal tract, kidney, respiratory system and platelets when these drugs inhibit COX-2 at the inflammatory site to contribute their therapeutic, beneficial effects (Mitchell & Warner 1999, Ruoff 1998, Sousa *et al.* 1997). Since diseases are mainly related to COX-2, selective COX-2 inhibitors have been developed and introduced to clinical treatment which offer real hope for safer anti-inflammatory drugs although their long-term safety and efficacy as questions remain unanswered. Pratico *et al.* reported a 30% reduction in atherosclerosis by the COX-2 inhibitor, nimesulide (Pratico *et al.* 2001). In a low-density lipoproteins receptor-deficient (LDLR-/-) mouse model, the atherosclerosis was reduced by the COX-2 inhibitor, rofecoxib (Burleigh *et al.*

2002). Clinically, administration of rofecoxib lowers the levels of C-reactive protein (CRP) and interleukin-6 (IL-6) in patients with ACS, which may lead to the retardation of coronary atherosclerosis and coronary events (Monakier *et al.* 2004). COX inhibitors also slow down the progression of Alzheimer's disease and inhibit the growth of cancers (Hull *et al.* 2000, Hull *et al.* 2002, Lin *et al.* 2002, Souza *et al.* 2000, Xiang *et al.* 2002). Nevertheless, the roles of specific COX inhibitors in cardiovascular disease are still under debate. Some studies have even pointed out that the use of selective COX-2 inhibitors can increase the risk of myocardial infarction (Andersohn *et al.* 2006, Caldwell *et al.* 2006, Gislason *et al.* 2006, Kearney *et al.* 2006). Rofecoxib was removed from the US market in 2004 due to increased cardiovascular risk. Another selective COX-2 inhibitor, celecoxib, has also been warned to have an increased cardiovascular risk. On the other hand, some reports mentioned that the elevated cardiovascular risk is not a drug class effect of selective COX-2 inhibitors (Spalding *et al.* 2007); between traditional NSAIDs and celecoxib there is no significant differences in the cardiovascular risk related to treatment (Huang *et al.* 2006, Velentgas *et al.* 2006), and COX-2 inhibitors perhaps are protective at low doses and risk-inducing only at higher doses (McGettigan *et al.* 2006). These reports suggested that the elevated cardiovascular risk is associated with higher doses (McGettigan *et al.* 2006), hypertension (Spalding *et al.* 2007), history of myocardial infarction (Brophy *et al.* 2007) and pre-existing medical conditions (Huang *et al.* 2006) rather than COX-2 inhibitors, especially celecoxib. The roles of COX-2 and its selective inhibitors are quite complex in atherosclerosis and this has attracted more and more attention for investigation and discussions (Cuccurullo *et al.* 2007a, Cuccurullo *et al.* 2007b, Iezzi *et al.* 2007, Salinas *et al.* 2007). For safety reasons, selective COX-2 inhibitors should not be used for patients with atherosclerosis.

2.3.3 *Rapamycin*

Rapamycin (rapamune, sirolimus) is mainly used clinically in organ transplantation to inhibit the immune response with its immunosuppressive effect for the prophylaxis of allograft rejection.

Rapamycin is one of macrolide antibiotics with immunosuppressive, antiproliferative, and antimigratory properties (Marx *et al.* 1995, Poon *et al.* 1996) which reverses the acute rejection of heart, kidney and pancreas allograft (Chen *et al.* 1993). It is produced by *Streptomyces hygroscopicus,* a streptomycete isolated from a soil sample from Easter Island (Rapa Nui) (Sehgal *et al.* 1975). The cellular

actions and immunosuppressant activity of rapamycin are mediated by binding to its intracellular receptor, the FK506 binding protein (FKBP12), and blocking signals induced by the mammalian target of rapamycin (mTOR) and arresting cell cycle progression in T lymphocytes from G1 to S phase transition (Morice *et al.* 1993, Poon *et al.* 1996). Because of its properties and the unique mechanism of action, rapamycin is applied in transplantation and other areas where cell proliferation plays a key role. Rapamycin and FK506 have similar structures in their binding domains, and they share a family of immunophilins called FK506 binding proteins (FKBPs). They are supposed to exert their immunosuppressive effects through the inhibition of the peptidyl prolyl cis-trans isomerase activity inherent in the immunophilins (Fretz *et al.* 1991, Harding *et al.* 1989). Among immunophilins, only rapamycin possesses both antiproliferative and antimigratory properties; and therefore rapamycin is used to prevent rejection in transplanted hearts and prevent restenosis in coronary stenting (Marx *et al.* 1995, Poon *et al.* 1996).

Recent studies have shown that rapamycin-coated eluting-stents can inhibit intimal thickening in patients with coronary artery disease (Gregory *et al.* 1993, Morris *et al.* 1995) to prevent neointima formation and to reduce the risk of in-stent restenosis (Eisenberg & Konnyu 2006). Rapamycin has been shown to reduce atherosclerotic lesion size in apoE-null mice (Castro *et al.* 2004, Elloso *et al.* 2003). Neointimal proliferation is the principal mechanism underlying in-stent restenosis, and it is the result of endothelial damage after stent expansion. The neointimal response is characterized by the proliferation and migration of smooth muscle cells as well as the deposition of the extracellular matrix. Compared with bare metal stents, stents coated with antiproliferative agents such as rapamycin have shown remarkable promise at reducing the rates of restenosis, and there has also been an explosive growth in their use recently (Eisenberg & Konnyu 2006). Rapamycin might exert its anti-atherosclerotic effects on vascular smooth muscle cells (VSMCs) by inhibiting mRNA and protein expression of inflammatory cytokines IL-6, tumor necrosis factor-alpha (TNF-α), IL-8, monocyte chemoattractant protein-1, inhibiting lipid uptake together with increasing cholesterol efflux (Ma *et al.* 2007). In apoE knockout mice, 12 weeks of treatment with sirolimus (rapamycin) reduced the cholesterol content of the aortic arch by 36% which is obviously beneficial for atherosclerosis (Basso *et al.* 2003). Destabilized atherosclerotic plaques induce the acute coronary syndromes and sudden death. Macrophages play a key role in plaque destabilization. A study with everolimus, a derivative of rapamycin (sirolimus) with similar effect, demonstrated selective

clearance of macrophages in atherosclerotic plaques by autophagy may be another novel mechanism of anti-atherosclerotic effects of rapamycin (Verheye *et al.* 2007). Autophagy is one of the cell death mechanisms in VSMCs and is involved in the progression of atherosclerosis. Kamada *et al.* reported the relationship between mTOR and autophagy and suggested that the TOR-ATG1, one of the autophagy-related ATG genes, pathway might be conserved in eukaryotic cells (Kamada *et al.* 2000).

Moreover, the effects and clinical treatment of rapamycin extend to the treatment of ocular ischemia (Nussbaumer-Ochsner *et al.* 2006), acute lymphoblastic leukemia (Avellino *et al.* 2005), to the reduction of the growth of Epstein Barr virus B-cell lymphomas (Nepomuceno *et al.* 2003) and Kaposi sarcoma (Di Paolo *et al.* 2007). Rapamycin is also an alternative drug in patients with immunosuppressant calcineurin inhibitors associated neurotoxicity (Daoud *et al.* 2007).

Several recent studies have shown that *C. pneumoniae* infection is an additional factor for chronic allograft rejection (Kwiatkowski *et al.* 2006) and may lead to complications and worsen the prognosis in lung (Glanville 2001, Glanville *et al.* 2005, Kotsimbos *et al.* 2005), liver (Lotz *et al.* 2004), and heart transplantation (Pieniazek *et al.* 2003, Subramanian *et al.* 2002). A serological study also suggested that *C. pneumoniae* could play an important role in the mechanism of in-stent restenosis (Hayashida *et al.* 2002). It has been shown earlier that rapamycin can inhibit the infectivity of *C. trachomatis* (Lundemose *et al.* 1993), but whether it has same effects on *C. pneumoniae* is still unclear. If it possesses antichlamydial properties, the beneficial effects of rapamycin in the treatment of the acute rejection of allograft and in-stent restenosis may be partly due to its antichlamydial activities.

2.3.4 Other drugs

Some other existing drugs have been shown to have beneficial effects on cardiovascular diseases.

Verapamil

Verapamil is used to treat hypertension and arrhythmias. It induces peripheral vasodilation, thus decreasing the loads to the heart and increases the blood supply to heart and peripheral tissues.

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Verapamil is an α_1-adrenoceptor antagonist, L-type calcium channel blocker which inhibits the transmembrane calcium ion influx to myocardium and blood vessels. It selectively blocks only the slow calcium channel and does not affect the fast channel, thus reducing the free calcium ions in the cells of myocardium and blood vessels. During angioplasty of acute myocardial infarction, vearpamil showed its beneficial effect on attenuating microvascular dysfunction and improving myocardial blood flow (Hang *et al.* 2005, Taniyama *et al.* 1997). For late in-hospital phase patients with acute myocardial infarction with or without heart failure, verapamil might improve the prognosis and reduces mortality and morbidity (Jespersen 1999). Animal experiments revealed that the cardioprotective effect of verapamil may not only depend on the blockage of the calcium channel but also the action on the sarcoplasmic reticulum (ryanodine sensitive calcium channel) or the mitochondria (Paquette *et al.* 1999).

In the treatment of cardiovascular diseases, in addition to common hypertension and arrhythmias, verapamil also diminishes the endothelial injury in diabetic hypertensive rats (Conrad *et al.* 1994). Since calcium antagonist verapamil is an antihypertensive agent, the question is raised whether its antiatherosclerotic effect is due to the reduction of the blood pressure or is independently of its antihypertensive effect. Studies in animals have reported the verapamil can effectively prevent or slow the progression of atherosclerosis but it cannot, or can only ineffectively, cause regression of atherosclerosis (Stein *et al.* 1985), so that it has a preventive antiatherosclerotic effect rather than therapeutic one. Verapamil prevents or slows the atherosclerosis by inhibiting a calcium overload and by interfering with lipid metabolism and deposition but not by lowering the blood pressure (Blumlein *et al.* 1984).

There are also reports about antibiotic effects of verapamil. It inhibits the replication of cytomegalovirus (Albrecht *et al.* 1987), influenza A virus (Nugent & Shanley 1984) and measles viruses (Shainkin-Kestenbaum *et al.* 1993). Moreover, verapamil was reported that it could inhibit the growth of *C. trachomatis* in host cells by inhibiting the differentiation of chlamydial RBs into EBs (Shainkin-Kestenbaum *et al.* 1989). It implicated the possibility for verapamil to inhibit the growth of *C. pneumoniae*.

Statins

Statins are 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase (HMGR) inhibitors. They lower cholesterol biosynthesis by competitively

inhibiting the conversion of acetyl CoA and acetoacetyl CoA to mevalonate and by preventing the formation of the isoprenoids (Istvan 2002). Because of their powerful cholesterol lowering effect, they are prescribed extensively in the primary (Downs *et al.* 1998) and secondary (Pedersen *et al.* 2005) prevention of cardiovascular disease. In humans, low-density lipoprotein-cholesterol (LDL-C) is intrinsically linked to atherothrombosis and its oxidized form, oxidized LDL-C (ox-LDL-C), has some deleterious effects. Therefore reductions of the LDL-C in circulation and the degree of oxidation should be beneficial for patients. A meta-analysis of several statins (pravastatin, simvastatin, atorvastatin, rosuvastatin, fluvastatin and lovastatin) confirmed the significant positive relationship between the reduction in LDL-C by statins and the reduction in the risk for major cardiovascular events (Delahoy *et al.* 2009). Statins exert their beneficial effects on cardiovascular disease not only by their cholesterol lowering effects, but also by their cholesterol-independent or pleiotropic effects. Statins can improve endothelial function by increasing NO, which gives the vessel wall its vasodilator and antithrombotic properties (Pearson 1999) and bioavailability (Laufs *et al.* 1998). Statins are also involved in attenuating vascular remodeling, inhibiting vascular inflammatory response and stabilizing atherosclerotic plaques (Assmus *et al.* 2003, Dichtl *et al.* 2003, Inoue *et al.* 2000). An even more interesting thing is the antichlamydial effect of statins. In addition to the indirect inhibitory effect of cerivastatin on *C. pneumoniae* infection (Dechend *et al.* 2003, Kothe *et al.* 2000), Erkkilä *et al.* reported that simvastatin treatment has an effect on the microbe itself, reducing viable *C. pneumoniae* counts in mice (Erkkila *et al.* 2005).

β-blockers

β-adrenergic blockers are important agents in the treatment of cardiovascular diseases. Studies have shown that β-blockers can effectively save patients from sudden cardiac death after myocardial infarction, and reduce recurrent myocardial infarction and total mortality in patients with myocardial infarction (Olsson *et al.* 1985, Wikstrand & Kendall 1992). Improving myocardial function, reducing myocardial oxygen consumption, and reducing blood pressure and antiarrhythmia may be the mechanisms by which β-blockers reduce the risk of myocardial infarction. Recent study showed cardioselective β-blockers could decrease mortality in patients with chronic obstructive pulmonary disease (COPD); and accumulated evidence also suggests that β-blockers have antiatherosclerotic effects and can slow the progression of coronary atherosclerosis (Sipahi *et al.* 2007, van

Gestel *et al.* 2008, Wikstrand *et al.* 2003). An animal experiment showed that nipradilol, a nitric oxide-releasing β-blocker, successfully restored the nitric oxide bioavailability and possible interaction with oxygen radicals, and thus exerted an anti-atherosclerotic effect (Thakur *et al.* 2002). Similar results were obtained in another experiment where nebivolol inhibited the development of atherosclerosis. In this experiment nebivolol increased plasma nitrates, endothelial reactivity and aortic eNOS expression (de Nigris *et al.* 2008). Obviously, nitric oxide (NO) is beneficial to the vessels in atherosclerosis.

ACE inhibitors and ARBs (or AT blockers)

The angiotensin-converting enzyme (ACE) catalyzes the generation of angiotensin II. Angiotensin II mediates the activation of the renin-angiotensin system (RAS) which is implicated in many types of cardiovascular disease: hypertension, acute myocardial infarction and atherosclerosis (Smith *et al.* 2001). ACE inhibitors and angiotensin receptor blockers (ARBs or AT blockers) have anti-inflammatory properties. They are used in coronary heart diseases, atherosclerosis, nephritis, diabetes, diabetic nephropathy, scleroderma, arthritis and congestive heart failure (Egido & Ruiz-Ortega 2007, Ram 2008, Silverstein & Ram 2005). ACE inhibitors can restrict the vascular remodeling processes during vascular injury, atherosclerosis, and aneurysm formation (Heeneman *et al.* 2007), and have protective effects on aortic rupture (Hackam *et al.* 2006). ARBs block the angiotensin II receptor type 1 causing vasodilation directly thus releasing the symptoms caused by vasoconstriction. Nevertheless the beneficial effects of ARBs in reducing cardiovascular mortality and morbidity are not only dependent on their vascular effects but also, at least partly, dependent on their anti-inflammatory and metabolic effects (Barra *et al.* 2009). Substance-specific differences between individual ACE inhibitors or ARBs can cause different results (Friedrich *et al.* 2006, Pantev *et al.* 2002).

3   Aim of the study

The principal aim of this study was to investigate the antichlamydial effect of several drugs used for cardiovascular diseases by using the cell culture method. The specific aims were:

1. To evaluate the experimental conditions of cell culture studies, which may affect the infectivity of *C. pneumoniae* during the study processes.
2. To investigate how the heparan sulfate-like glycosaminoglycans can influence the infectivity of *C. pneumoniae*.
3. To investigate the effect of selective cyclooxygenase inhibitors on the growth of *C. pneumoniae*.
4. To investigate the effect of rapamycin on inhibiting the development of *C. pneumoniae*.

4   Materials and methods

4.1   Culture medium

The basic culture medium used in this study is the RPMI 1640 tissue culture medium (Sigma, St. Louis, MO, USA) supplemented with 7.5% heat inactivated fetal calf serum (FCS) (BioClot (Pty) Ltd), 2 mM glutamine, and 20 µg/ml streptomycin which is used for the propagation of host cells and the culture of uninfected cells. For culturing infected cells, the culture medium is supplemented with an extra 0.5 µg/ml of cycloheximide except where specifically mentioned.

4.2   Host cells

The human originating epithelial cells, more specifically the HL cell line (Cles & Stamm 1990, Kuo & Grayston 1990) was used. HL cells were cultured in the medium mentioned above in a 5% CO_2 atmosphere at 37 °C until used.

4.3   *Chlamydia* strains

C. pneumoniae Kajaani 7 (K7) (Ekman *et al.* 1993) and Parola strains, originally isolated from Finnish patients with acute respiratory infection, were propagated in HL cell monolayers. Infected cells were harvested with sterile glass beads and ultrasonically disrupted. Cell debris was separated with low-speed centrifugation, followed by sonication and two cycles of high-speed centrifugation through Urografin gradients to purify chlamydial particles. Finally, the pellet obtained was resuspended in a sucrose-phosphate-glutamic acid buffer (SPG: 0.2 M sucrose, 3.8 mM KH_2PO_4, 6.7 mM Na_2HPO_4, 5 mM L-glutamic acid, pH 7.4) and stored at −70 °C in aliquots until used. Other chlamydial strains used in this study include *C. pneumoniae* CWL029 (VR-1310), *C. trachomatis* serovars E (VR348B) and L2 (VR-902B), which were obtained from the American Type Culture Collection (ATCC). They were propagated and purified in the same way as *C. pneumoniae* K7.

4.4   Reagents

The drugs used in this study are listed in Table 5:

Table 5. Drugs and their manufacturers.

Drugs	Manufacturer
heparin (H-3149)	Sigma, St. Louis, MO, USA
enoxaparin (RP 54563A, Batch:WSD 3014)	Aventis Pharma, France
low molecular weight heparin (H-3400)	Sigma, St. Louis, MO, USA
chondroitin sulfate A (C-8529)	Sigma, St. Louis, MO, USA
heparinase I (H-2519)	Sigma, St. Louis, MO, USA
COX-1 inhibitor SC-560	Calbiochem, Darmstadt, Germany
COX-2 inhibitor PTPBS	Calbiochem, Darmstadt, Germany
rapamycin R0395	Sigma-Aldrich, USA

The drugs were dissolved in either PBS pH 7.2 or dimethylsulfoxide (DMSO) to make the stock solutions and stored according to the manufacturer's instructions.

4.5 Evaluation of experimental conditions in cell cultures (I)

In order to exclude the methodological factors which affect the infectivity of *C. pneumoniae* in cell cultures, some experiments have been performed to evaluate the impact of experimental conditions (I).

To evaluate the effect of FCS and temperature, the inoculation of chlamydial EBs and the pre-treatment of the EBs with heparin and heparinase were performed in either a FCS-free PBS or culture medium with 7.5% FCS, at either 4 °C or 35 °C.

A 15-s sonication in a Bransonic 42 bath sonicator, or without sonication, was performed to chlamydial EBs before or after heparin pre-treatment to investigate the effect of sonication. After the pre-treatment of chlamydial EBs with different drugs, the chlamydial EBs were washed with ice-cold PBS.

In this study, chlamydial EBs pre-treated with drugs were generally washed once before being inoculated into HL cells. In order to know the impact of washing, 9×10^4 untreated IFUs were washed with ice-cold PBS once or left unwashed and inoculated into HL cell monolayers.

4.6 Infection assays

The similar infection methods were used in different studies. In principle, approximately $3–3.5 \times 10^5$ HL cells were placed on 13 mm diameter sterile glass

coverslips in 24-well culture plates (NUNC) and were allowed to grow for 24 h to reach confluent cell monolayers before infection. After the confluent cell monolayers were washed with ice-cold PBS, all wells of the culture plates were inoculated with the same amount of chlamydial EBs by centrifugation at $480 \times g$ for 1h or incubated at 4 °C for 2 h without centrifugation. Depending on the experiment, 300–500 inclusions, at least 200 inclusions, were expected to achieve on each coverslip from the inoculated chlamydial EBs. Then the cell monolayers were washed three times with ice-cold PBS, and incubated in 1 ml of a culture medium supplemented with 0.5 µg/ml of cycloheximide for 48 h (*C. trachomatis*) or 72 h (*C. pneumoniae*) at 35 °C and in a 5% CO_2 atmosphere.

4.6.1 Applying drugs to HL cells before infection

To investigate the effect of pretreatment of host cells with drugs on the infectivity of *C. pneumoniae*, the confluent HL cell monolayers were washed with ice-cold PBS and incubated in the culture medium containing 1U/ml of heparinase I, 500 µg/ml of heparin (I, II), chondroitin sulfate A (CSA), enoxaparin and low molecular weight heparin (LMWH) respectively (II), or 23 µg/ml of rapamycin (IV). The cell monolayers in a medium containing heparin, CSA, enoxaparin and LMWH were incubated at 4 °C for 1h, and those in a medium containing heparinase I and rapamycin at 35 °C for 1h. Then they were washed twice with ice-cold PBS, inoculated with chlamydial EBs with (IV) or without (I, II) centrifuge, washed and incubated in 1 ml of a culture medium supplemented with 0.5 µg/ml of cycloheximide for 48 h or 72 h as described above. In order to know the effect of FCS, this pretreatment with heparin and heparinase I was performed also in PBS instead of culture medium (I).

4.6.2 Applying drugs to chlamydial EBs before infection

Chlamydial EBs were pretreated by being suspended in 1U/ml of heparinase I, 500 µg/ml of heparin diluted in PBS (I) or culture medium (I, II), and 50 µg/ml, 5 µg/ml, 0.5 µg/ml, 0.05 µg/ml, 0.005 µg/ml of heparin, enoxaparin, LMWH and CSA diluted in culture medium respectively (II), or 23 µg/ml of rapamycin diluted in culture medium (IV). The suspensions were kept at 4 °C for 1h except the suspensions with heparinase I and rapamycin were at 35 °C for 1h. The pretreated chlamydial EBs were washed with ice-cold PBS and resuspended in a culture

medium or PBS, then inoculated into the washed untreated HL cell monolayers with (IV) or without (I, II) centrifugation.

4.6.3 Applying drugs to chlamydial EBs and HL cells during inoculation

In some experiments, 500 µg/ml, 50 µg/ml, 5 µg/ml, 0.5 µg/ml, 0.05 µg/ml or 0.005 µg/ml heparin, 500 µg/ml CSA, enoxaparin or LMWH (II) or selective COX inhibitors SC-560 or PTPBS at concentrations of 0.01–20 µg/ml were present during inoculating chlamydial EBs into HL cells (III).

4.6.4 Applying drugs to infected cells after inoculation

In order to investigate the effect of selective COX inhibitors and rapamycin on the growth of *C. pneumoniae*, SC-560 or PTPBS were present in the culture medium containing 0.5 µg/ml cycloheximide at concentrations of 0.01–20 µg/ml respectively after inoculation. Rapamycin was present in the culture medium after inoculation, at concentrations of 23 µg/ml, 11 µg/ml, 7 µg/ml or 3.5 µg/ml respectively, for either 8-hour periods, 0–8 h, 8–16 h, 16–24 h or 24–32 h, or 0–48 h (*C. trachomatis*) or 72 h (*C. pneumoniae*) continuously.

4.6.5 Recovery of C. pneumoniae after treatment

In the studies with selective COX inhibitors and rapamycin, the culture medium contained corresponding drugs during 72 h incubation after inoculation. The viability and recoverability of newborn chlamydial EBs in such incubation were studied by extending the incubation in fresh culture medium without any COX inhibitors for another 72 h (III) and by re-passaging the infected cells with *C. pneumoniae* into fresh HL cell monolayers with centrifuge and then incubating the repassaged cells in the medium without any drugs for 72 h at 35 °C and 5% CO_2 (III, IV).

4.7   16S rRNA real-time polymerase chain reaction (PCR) (II, IV)

DNA of *C. pneumoniae* was extracted with the DNA purification Kit (Qiagen, Hilden, Germany) from the diluted stock of *C. pneumoniae* (II) or cultured HL cells infected by *C. pneumoniae* (IV). A LightCycler instrument (Roche Diagnostics

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GmbH, Rotkreuz, Switzerland) was employed for quantitative real-time analysis of chlamydial 16S rRNA. For 16S rRNA gene of *C. pneumoniae*, the LightCycler PCR reaction contained 10 pmol/μl of each primer and 4 pmol/μl of each specific hybridization probe. The sequence of forward primer CpnA is 5'-TGA CAA CTG TAG AAA TAC AGC-3', and of reverse primer CpnB is 5'-CGC CTC TCT CCT ATA AAT-3' (Gaydos *et al.* 1992). The sequence of probe CP16FL is 5'-GTA GCA AGA TCG TGA GAT GGA GCA A-FL-3', and of probe CP16LC is 5'-Red 640-TCC TAA AAG CTA GCC CCA GTT C-Ph-3' (Reischl *et al.* 2003). *C. pneumoniae* genome counts obtained from different wells of the culture plate were compared with each other or with the numbers of inclusions in cultures.

4.8   Quantification of COX-1 and COX-2 mRNA expression

HL cell monolayers in a 24-well culture plate were inoculated with chlamydial EBs (approximately 3–4 inclusion-forming units/cell) by centrifuging for 1 h accompanied with or without 7 μg/ml SC-560 or 12 μg/ml PTPBS. Then, the cell monolayers were washed with ice-cold PBS and incubated for 6 h in the culture medium containing corresponding selective COX inhibitors as in inoculation but without cycloheximide. With RNA*later* RNA Stabilization Reagent (Qiagen) both infected and uninfected cells were collected.

Table 6. Primers and probes used in LightCycler PCR for quantification of COX-1 and COX2 mRNA expression.

Primer / probe	COX-1	COX-2	PBGD*
Forward primer	5'-CCAGGAGCTCGTAGG AGAGAAG-3'	5'-CGACTCCCTTGGGTGTCA AAGGTAA-3'	5'-AGAGTGATTCGCGTG GGTACC-3'
Reverse primer	5'-GTGGCCGTCTTGACA ATGTTAA-3'	5'-AAAGACCTCCTGCCCCAC AGCAA-3'	5'-GGCTCCGATGGTGA AGCC-3'
Probes			
Fluorescein	5'-GCACTTTTCAAGAAGC AGTCCAGGGTAG-FL-3'	5'-CCTCCCACAGTCAAAGATA CTCAGGCAGAGATG-FL-3'	5'-AGTGGACCTGGTTGT TCACTCCTTGAA-FL-3'
Lc-Red 640	5'-LC Red640-ACTCCAAC GCATCAATGTCTCCATA CAA-ph-3'	5'-LC Red640-CTACCCTCCTC AAGTCCCTGAGCATCTACG-p h-3'	5'-LC Red640-ACCTGCC CACTGTGCTTCCTCCT- ph-3'

*Housekeeping porphobilinogen deaminase gene.

Total RNA of these cells were isolated with RNase-Free DNase Set and RNeasy Kit (Qiagen) according to the instructions of manufacturer. With the Omniscript RT Kit (Qiagen), cDNA was constructed by reverse transcription of the isolated RNA.

Quantification of COX-1 and COX-2 mRNA expression was performed by real-time PCR with a LightCycler instrument (Roche Diagnostics). The PCR reaction contained 5 mM of $MgCl_2$, 0.5 μM of the primers and 0.2 μM of the probes given in Table 6, and 2 μl of reverse-transcribed cDNA template in a total volume of 20 μl and was performed using a Roche LightCycler FastStart DNA Master Hybridization Probes Kit. The amplification cycle consisted of 5 s at $95^{o}C$, 12 s at 65 °C and 12 s at 72 °C. The COX-1 and COX-2 mRNA levels were normalized using the housekeeping gene *PBGD* expression as reference. These ratios were further calibrated with the corresponding normalized ratio from uninfected, untreated control cells. With purification, amplification and ethanol precipitation of the correct PCR product from agarose gel, the specific standards for each gene were produced. Analyses of the expressions of COX-1 and COX-2 mRNA were done using the LightCycler Relative Quantification-Monocolor method with external standards (LightCycler software version 4.05).

Each abovementioned (4.5–4.8) experiment or test was repeated at least three to ten times and duplicates or triplicates were done.

4.9   Transmission electron microscopy

For transmission electron microscopy, the monolayers of HL cells were infected with 4×10^6 IFU, approximately 1 IFU/cell, of *C. pneumoniae* CWL029 and cultured in a medium containing 11 μg/ml of rapamycin for 72 h. Some monolayers cultured in a culture medium without rapamycin were set as a control. Then, the infected HL cells were fixed in 2.5% glutaraldehyde in 0.1 M sodium phosphate buffer (pH 7.4), detached with a plastic cell scraper, pelleted, postfixed in 1% osmium tetroxide, and embedded in Epon Embed 812. Thin sections were cut with a Reichert Ultracut ultramicrotome and examined under a Philips CM100 transmission electron microscope.

4.10   Analysis of chlamydial development

Infected cells, both from the first and second passages, were fixed with 100% methanol after 48 h or 72 h incubation and stained with a fluorescein isothiocyanate (FITC)-conjugated murine monoclonal antibody genus-specific to *Chlamydia*

(Pathfinder Chlamydia Culture Confirmation System; Bio-Rad S.A., Redmond, WA 98052, USA). All inclusions on the coverslips were counted under a fluorescent microscope (Axioskop 2, Carl Zeiss, Jena, Germany). The inclusion counts from the control infection were considered as 100% and were compared with the inclusion counts from other infections affected by the drugs. Morphological differences of the stained inclusions were examined under a confocal microscope (LSM510, Carl Zeiss (III) or Olympus Confocal Microscope Fluoview FV1000 (IV)).

4.11 Statistical analyses

Statistics was performed with SPSS statistical software (SPSS Inc., Chicago, IL). Wilcoxon's signed ranks test and the paired-samples *t*-test were used to compare the inclusion counts in test cells to those in control cells of the same strain and the expressions of COX-1 and COX-2 mRNA.

5   Results

5.1   Influences of experiment conditions on the infectivity of *Chlamydia*

5.1.1 The effect of FCS and temperature on chlamydial infectivity

Before this study, the medium for pre-treatment and inoculation was evaluated by testing the pre-treatment and inoculation in either PBS or culture mediums supplemented with 7.5% FCS. When pre-treatment for HL cells and inoculation were done at 4°C, there was no significant difference between in PBS and the culture medium with FCS, but if the pre-treatment was for chlamydial EBs in PBS compared to that in the culture medium with FCS, the infectivity of *C. pneumoniae* had decreased more than 90%: inclusion counts decreased from 364 ± 54.5 to 25 ± 1.5 ($p < 0.001$). When pre-treatment for HL cells was in PBS at 35°C, all cells on the glass coverslips detached, while cells in a culture medium with FCS remained normal. When the pre-treatment was done for chlamydial EBs at 35 °C, the infectivity of *C. pneumoniae* decreased more than 90% in the experiments in PBS compared to those in culture medium with FCS: inclusion counts decreased from 579 ± 6.9 in a culture medium to 22 ± 3.3 in PBS (untreated) or from 547 ± 2.2 in a culture medium to 58 ± 14.1 in PBS (treated) ($p < 0.001$). If instead of PBS, a FCS-free culture medium was used, the same results were obtained.

5.1.2 Sonication

The 15 s sonication tested in this study did not show any impact on the infectivity of *C. pneumoniae*, no matter if it was done before or after pre-treatment, with or without pre-treatment. Prolonged sonication time of 5–10 min decreased the inclusion counts by 30–40%.

5.1.3 Washing

The testing of washing of chlamydial EBs before inoculation showed that this procedure could increase the infectivity of *C. pneumoniae* significantly. Comparing the inclusion counts on the glass coverslips, the inclusion counts from

unwashed chlamydial EB were 163 ± 67 while those from washed EBs were 577 ± 371 (n = 15 for both, $p < 0.001$).

5.2 Effects of drugs on inclusion counts

5.2.1 Pretreatment of HL cells

The outcome of the treatment of HL cells with different selected drugs before infection was dependent both on the drugs and chlamydial strains. Generally, the inhibitory effect of GAGs was stronger for *C. pneumoniae* than for *C. trachomatis*. The effects of the members of the heparin family were similar. Fig. 2 shows that in the HL cells pretreated with 500 μg/ml of heparin, the infectivity of *C. pneumoniae* CWL029 reduced to 19%, and those of K7 to 18% (II) ($p < 0.01$) or 11.5% (I) ($p < 0.05$), of Parola to 3% ($p < 0.001$) and of *C. trachomatis* either to 52% of L2 and 47% of E ($p < 0.05$). Two lower molecular weight heparins, enoxaparin and LMWH, were tested only with *C. pneumoniae* CWL029 and K7 and the effects of both drugs to both organisms were quite similar: reducing the infectivity to about 25%. CSA reduced the infectivity of *C. pneumoniae* CWL029, K7 and Parola to 54%, 45% and 63% respectively ($p < 0.05$) but no effect was seen on *C. trachomatis* L2 and *C. trachomatis* E although 10% of the infectivity of *C. trachomatis* E was reduced ($p > 0.05$). Pretreatment with heparinase I induced the decrease of infectivity of *C. pneumoniae* CWL029 and K7 to 61% and 54.2% (I) or 59% (II) ($p < 0.05$). The most effective target of heparinase I was *C. pneumoniae* Parola strain: 90% of its infectivity was inhibited ($p < 0.001$). With *C. trachomatis* L2 and E, the infectivity were reduced to 72% and 58% respectively ($p < 0.05$). Pretreatment of the HL cells with 23 μg/ml of rapamycin did not exhibit any inhibitory effect either on *C. pneumoniae* CWL029 or *C. trachomatis* L2, but their inclusion counts increased to 103% and 106% respectively ($p > 0.05$).
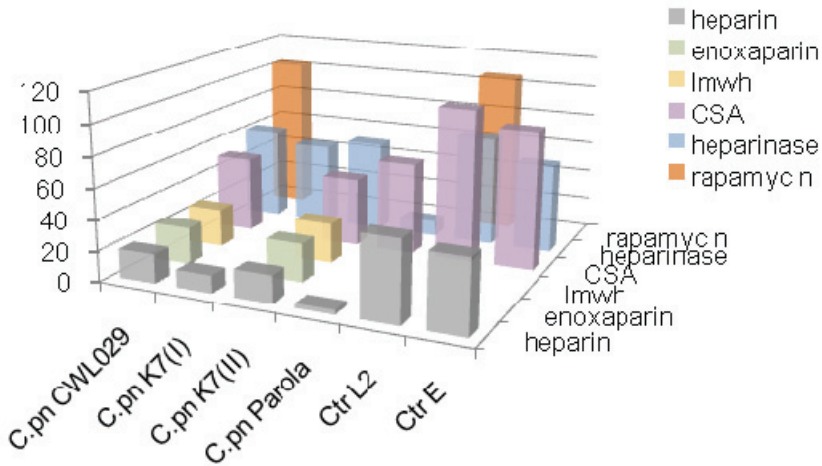
Fig. 2. Effect of pretreatment of HL cells with different drugs on the infectivity of different chlamydial strains.

5.2.2 Pretreatment of chlamydial EBs

The inhibitory effect of pretreatment of chlamydial EBs with different drugs on the infectivity or growth of chlamydiae varied between strains and between drugs as well as with the pretreatment of HL cells. The effect is shown in Fig. 3. All heparin, enoxaparin or LMWH preparations had similar inhibitory effects on the infectivity of each strain but different effects between strains. Relatively, *C. pneumoniae* K7 was the most sensitive to the heparin family. They caused the infectivity of *C. pneumoniae* K7 to be reduced by 40–70% (heparin 44.13% (II) – 70%(I); enoxaparin 41.63% and LMWH 55.74%) ($p < 0.05$). The other two *C. pneumoniae* strains, CWL029 and Parola, were not only unaffected by the heparins but their infectivity was enhanced by up to 30%. The infectivity of *C. pneumoniae* CWL029 was up to 119.28% (heparin), 108.82% (enoxaparin) and 123.24% (LMWH); and with *C. pneumoniae* Parola it was 117.46% (heparin), 101.13% (enoxaparin) and 131.01% (LMWH) ($p < 0.05$). For different *C. trachomatis* serovars, the inhibitory effects of the heparins were also different: heparin decreased the infectivity of *C. trachomatis* L2 to 49.3% but that of *C. trachomatis* E to 89.1% ($p < 0.05$); enoxaparin decreased the infectivity of *C. trachomatis* L2 to 80.2% but *C. trachomatis* E to only 43.6% ($p < 0.05$); and LMWH decreased that of *C.*

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trachomatis L2 to 74.12% and, similarly, that of *C. trachomatis* E to 89.5% ($p < 0.05$). The pretreatment of chlamydial EBs with CSA hugely increased the infectivity of *C. pneumoniae* strains: CWL029 up to 200.9% ($p < 0.05$), K7 to 127.7% ($p < 0.05$) and Parola to279.1% ($p < 0.01$), but decreased the infectivity of *C. trachomatis* E to 58.5% ($p < 0.01$) and was not effective on *C. trachomatis* L2 (106%, $p > 0.05$). Heparinase I digestion before infection induced the inhibition of the infectivity of *C. pneumoniae* CWL029 to 68.5% ($p < 0.05$) and *C. trachomatis* E to 21.2% ($p < 0.01$), but for *C. pneumoniae* K7, this inhibition was uncertain (94%, $p < 0.01$ (I); 94.5%, $p > 0.05$ (II)). For *C. trachomatis* L2, the digestion with heparinase I even enhanced the infectivity to 124.8% ($p < 0.05$). The pretreatment with rapamycin had only minor effect on the infectivity of *C. pneumoniae* CWL029 and *C. trachomatis* L2 decreasing them to 94.5% (CWL029) and 65% (L2) respectively ($p > 0.05$).
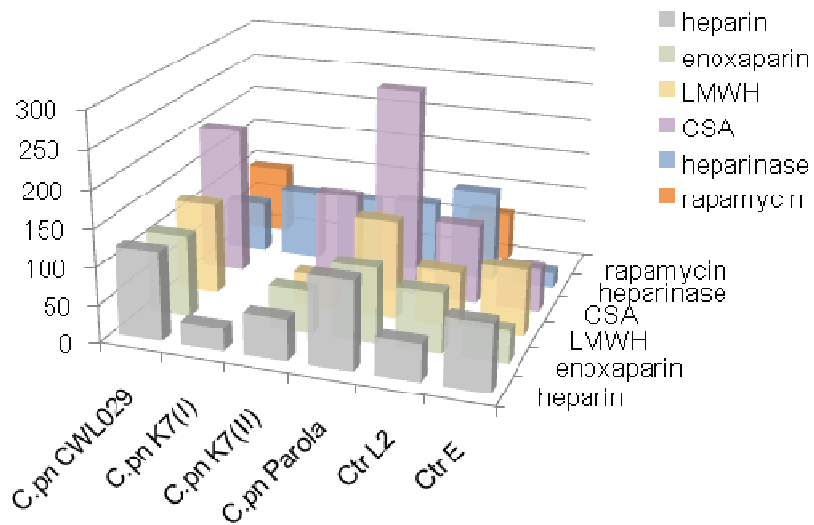

Fig. 3. Effect of pretreatment of chlamydial EBs with different drugs on the infectivity of different chlamydial strains.

5.2.3 Drugs present during inoculation

When heparin, enoxaparin, LMWH or CSA were present during inoculation, each one of them inhibited the infectivity of both the *C. pneumoniae* and *C. trachomatis* strains except that CSA had no inhibitory effect on the infectivity of *C. trachomatis* serovar L2 while heparin could almost totally inhibit it (Fig. 4). Heparin inhibited the infectivity of *C. pneumoniae* strains CWL029, K7, Parola and *C. trachomatis* strains serovar L2 and E to 17.5%, 18.7%, 24.1%, 3% and 14.7% respectively ($p < 0.05$); enoxaparin inhibited it to 29.5%, 35.1%, 29.2%, 12.6% and 14.5%; LMWH caused inhibition to 34.3%, 27.5%, 28.7%, 9.3% and 14.5% and CSA to 63.1%, 36.9%, 34.7%, 99.1% and 30.5% respectively ($p < 0.05$). The increase of concentration from 0.005 to 500 µg/ml of heparins inhibited the infectivity of *C. pneumoniae* in a dose-dependent manner ($p < 0.05$).
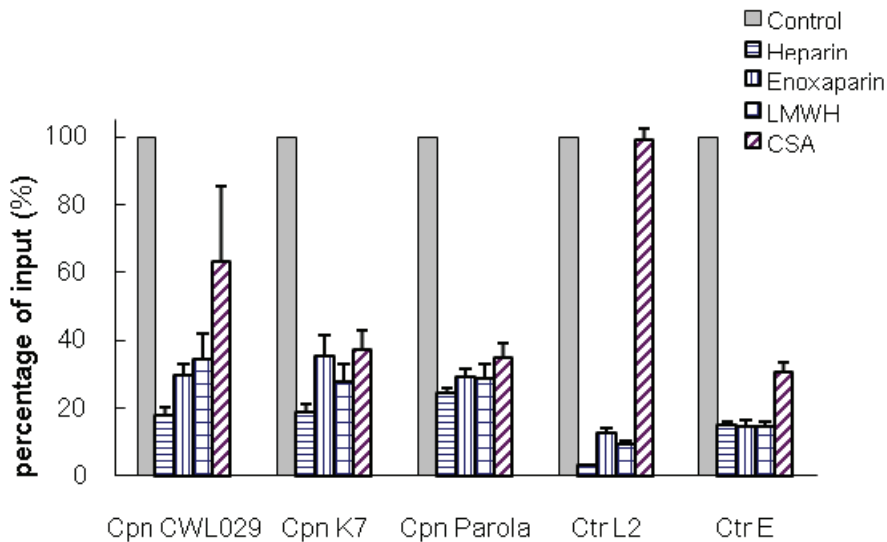
Fig. 4. Inhibitory effects of heparan sulfate-like GAGs on the infectivity of *C. pneumoniae* and *C. trachomatis* when they were present during inoculation.

5.2.4 Drugs present after inoculation

In this study, selective COX inhibitors were present both during and after inoculation of chlamydial EBs. 10 µg/ml of selective COX-1 inhibitor SC-560 or 18 µg/ml of selective COX-2 inhibitor PTPBS could totally inhibit the growth of *C. pneumoniae* K7 in HL cells. When the concentration of SC-560 varied within the

range of 4–9 µg/ml or that of PTPBS within the range of 2–16 µg/ml, they inhibited the growth of *C. pneumoniae* K7 concentration-dependently ($p < 0.05$). SC-560 in a concentration less than 0.01 µg/ml and PTPBS less than 0.05 µg/ml lost their inhibitory effect ($p > 0.05$) although 0.5 µg/ml of PTPBS could still cut down the growth of chlamydiae by 25.8% ($p < 0.05$).

A continuous presence of 23 µg/ml of rapamycin after inoculation caused the HL cell monolayers to detach from glass coverslips, but lower concentrations inhibited the growth of chlamydiae dose-dependently. 11 µg/ml of rapamycin reduced the growth of *C. trachomatis* by 76% ($p < 0.01$) and that of *C. pneumoniae* by 80% ($p < 0.01$), while the respective reductions by 7 µg/ml of rapamycin were 52% ($p < 0.01$) and 27% ($p < 0.05$). When the concentration of rapamycin was as low as 3.5 µg/ml, it no longer had a significant inhibitory effect on either *C. trachomatis* ($p > 0.05$) or *C. pneumoniae* ($p > 0.5$).

During the first 24 hours after inoculation, applying 11 µg/ml or 7 µg/ml of rapamycin in an 8-hour period manner did not show significant inhibitory effect on either *C. trachomatis* or *C. pneumoniae*, whereas 23 µg/ml of rapamycin inhibited the growth of both *C. trachomatis* and *C. pneumoniae* effectively at any 8-hour period of the first 24 hours: during 0–8 h, the inclusion counts of *C. trachomatis* were reduced by 81% ($p < 0.01$) and that of *C. pneumoniae* by 74% ($p < 0.01$); during 8–16 h, the inclusion counts of both *C. trachomatis* and *C. pneumoniae* were reduced by 94% ($p < 0.01$) and during 16–24 h, that of *C. trachomatis* by 71% ($p < 0.01$) and *C. pneumoniae* by 90% ($p < 0.01$). After the first 24 hour, the 8-hour period application of 23 µg/ml of rapamycin (24–32 h) had no more inhibitory effect on *C. pneumoniae* although it still reduced the inclusion counts of *C. trachomatis* by 64% ($p < 0.01$).

5.3   Effects of drugs on the morphology of inclusions

5.3.1 Confocal microscopy

With confocal microscopy, it was clear that the size of inclusions was affected by the drugs which caused smaller inclusions than those seen in the controls. The inclusions in the control infections were up to 11.5 ± 1.3 µm (II) – 12.6 ± 1.75 µm (III) in diameter, but in the presence of 9 µg/ml COX-1 inhibitor SC-560, 16 µg/ml COX-2 inhibitor PTPBS they were extremely small (1.9 ± 0.7 µm diameter (n = 13); $p < 0.001$) and disintegrated. At concentrations of < 4 µg/ml SC-560 and

< 2 µg/ml PTPBS, the inclusions were similar in size to those in the controls. When the infection was accompanied by 23 µg/ml of rapamycin during 8–16 h after inoculation, the size of inclusions was 3.4 ± 1.3 µm in diameter (n = 10); $p < 0.01$.

5.3.2 Transmission electron microscopy

The effects of rapamycin on the morphology of *C. pneumoniae* CWL029 inclusions were examined under a transmission electron microscope which showed that the inclusions were much smaller in the presence of rapamycin than in a normally grown inclusion. When infected HL cells' monolayers were cultured in the culture medium not containing any rapamycin, the inclusion was about 10–12 µm in diameter (Fig. 5a.) and the inclusions grown in the culture medium containing 11 µg/ml rapamycin for 72 h were only about 3.5 µm in diameter (Fig. 5b.). As shown in Fig. 5a., in normal inclusion there are more chlamydial particles and more matured EBs; whereas in Fig. 5b., in the inclusions under the influence of rapamycin, there are much fewer chlamydial particles and matured EBs. In the rapamycin treated inclusion (Fig. 5b.), there are also a few elongated reticulate bodies.

a)   b)
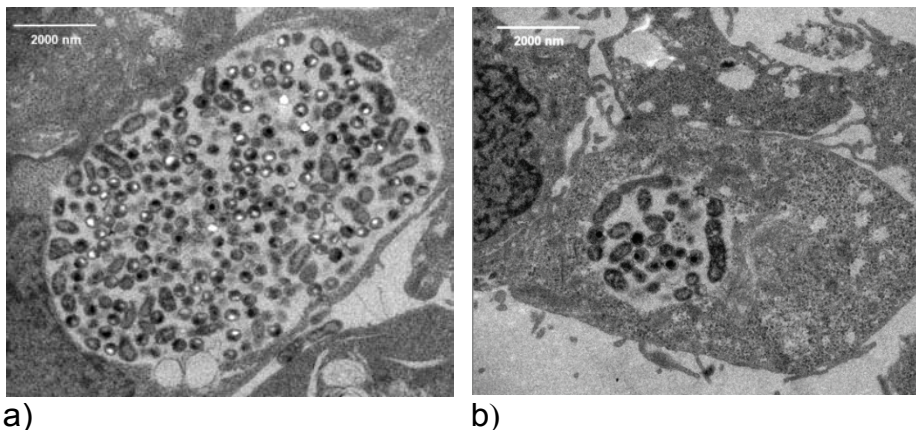
Fig. 5. Inclusions of *C. pneumoniae* CWL029 under a transmission electron microscope. A normal inclusion (a.) and an inclusion under the influence of 11 µg/ml of rapamycin (b.).

5.4   Recovery of *C. pneumoniae* after treatment

When 10 µg/ml SC-560 or 18 µg/ml PTPBS were present both during and after inoculation of chlamydial EBs, they could totally inhibit the growth of *C. pneumoniae* K7 in HL cells even extending the 72-h culture for another 72 h. No inclusions were observed on the stained glass coverslips. But if the COX inhibitors were removed after the first 72-h culture, a few inclusions could be seen after the extended culture and the size of the inclusions also returned to that seen in the control. With lower concentrations, the extended 72-h culture increased inclusion counts only two- to 3-fold (4 vs. 12 (7 µg/ml SC-560) or 28 vs. 73 (15 µg/ml PTPBS); $p < 0.01$) and the inclusions remained very small in size whereas inclusion counts significantly increased (about 5-fold (24 ± 3 vs. 113 ± 19; $p < 0.01$)) during the extended 72-h culture after the removal of the COX inhibitors compared with continued culture with the inhibitors and the size of inclusions recovered to that in the control.

   A few inclusions grew even from the cells treated with 10 µg/ml SC-560 or 18 µg/ml PTPBS during the first 72-h culture after repassaging the infected cells into fresh HL cell monolayers and incubating them in the medium without any COX inhibitors for 72 h at 35 °C and 5% CO_2. The size of the inclusions was similar to that of the control. Repassaging the cells infected with *C. pneumoniae* and grown in the medium containing 11 µg/ml rapamycin during the first passage showed the reduction of the inclusion counts to 57% at the first passage and to 24% at the second passage ($p < 0.05$) although rapamycin was absent during the second passage. The presence of 7 µg/ml of rapamycin at the first passage reduced the inclusion counts of *C. pneumoniae* significantly to 87.8% ($p < 0.05$) but did not affect the development at the second passage when rapamycin was absent. A lower concentration of rapamycin, for example 3.5 µg/ml, did not inhibit the chlamydial growth ($p > 0.05$) at either the first or second passage.

5.5   Quantification of chlamydial genomes

The main method used in this study to quantify the inhibitory effect of selected drugs on chlamydiae was to directly fix and stain the infected cells for counting chlamydial inclusions. The counting of inclusions is very subjective and inaccurate. Because chlamydial EBs tend to aggregate efficiently, it is impossible to know if an inclusion is caused by a clump of many EBs or by only a single EB. Additionally, purifying and washing EBs can cause significant loss of viability; and then they

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potentially confound the inhibitory results of the drugs. Hence, it is important to establish the reliable dispersion of chlamydial EBs in different preparations and to measure the viability relative to the total number of chlamydial particles used. With the LightCycler system, the gene for *C. pneumoniae* 16S rRNA was quantitatively analyzed by real-time PCR to measure chlamydial genomes in infected cells in relation to the total number of inoculated chlamydial EBs. The genome copy number of *C. pneumoniae* 16S rRNA fragments of 300 μl diluted chlamydial stock was 1023 (SD ± 393), and the average inclusion counts obtained from the coverslip inoculated with same amount chlamydial stock and cultured for 72 h was 323 (SD ± 39), thus the viability of chlamydial EBs in this preparation was about 37% (SD ± 17) (II). Under the influence of rapamycin, the chlamydial genome copy counts decreased from $6.01 \times 10^6 \pm 6.9 \times 10^5$ in control cells to $3.27 \times 10^6 \pm 3.9 \times 10^5$ in the cells treated with 11 μg/ml of rapamycin and thus genome counts decreased about 46% ($p < 0.05$), $8.0 \times 10^6 \pm 8.3 \times 10^5$ ($p = $ NS) in the cells treated with 7 μg/ml of rapamycin and $7.78 \times 10^6 \pm 8.2 \times 10^5$ ($p = $ NS) in the cells treated with 3.5 μg/ml of rapamycin (IV).

5.6 COX-1 and COX-2 mRNA expression

The expression levels of COX-1 and COX-2 mRNA were normalized using the housekeeping gene *PBGD* expression as a reference and analyzed using the LightCycler Relative Quantification-Monocolor method with external standards. From the control cells, which were uninfected with *C. pneumoniae* and untreated with any COX inhibitors, the normalized expression ratios of COX-1 and COX-2 mRNA were 1. As shown in Fig. 6, by calibrated with the normalized expression ratios of COX-1 and COX-2 of the control cells, the expression ratio of COX-1 mRNA in infected cells (untreated with COX-1 inhibitor) was 1.32, which in infected and COX-1 inhibitor treated cells was 0.8, and in uninfected but COX-1 inhibitor treated cells was 0.94 respectively. There was no significant difference between these ratios ($p > 0.05$). For COX-2 mRNA, the normalized expression ratios in infected, infected and COX-2 inhibitor treated, uninfected but COX-2 inhibitor treated cells were 0.64, 0.55 and 0.43. All ratios of COX-2 mRNA were significantly different from each other ($p < 0.05$) except between the ratios of infected and uninfected but COX-2 inhibitor treated cells ($p > 0.05$). It can be seen that both *C. pneumoniae* and selective COX inhibitors can downregulate the COX-2 mRNA expression of HL cells but cannot up- or downregulate the expression of COX-1 mRNA in the HL cells.
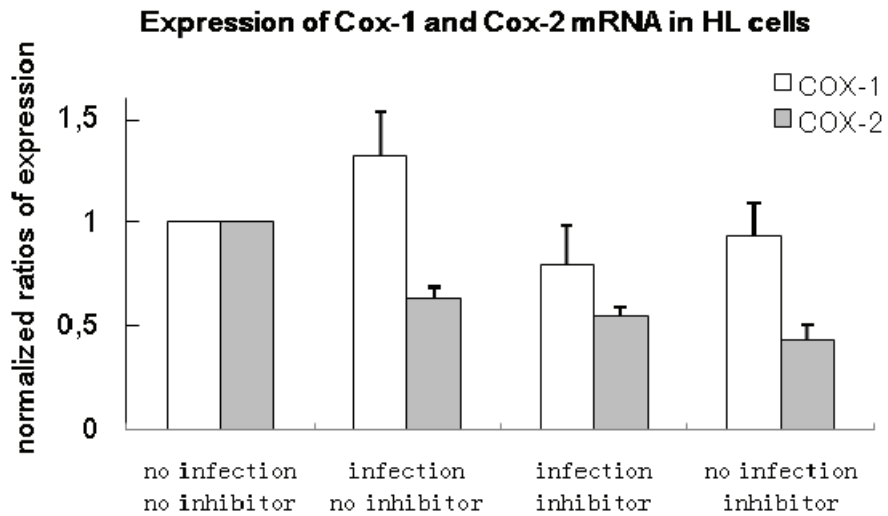
Fig. 6. Expression of COX-1 and COX-2 mRNA in HL cells. Measurements were from uninfected HL cells, HL cells infected with *C. pneumoniae* K7, HL cells infected with *C. pneumoniae* K7 and incubated with COX inhibitors (7 µg/ml SC-560 or 12 µg/ml PTPBS), and uninfected HL cells with COX inhibitors (7 µg/ml SC-560 or 12 µg/ml PTPBS). There was no significant difference in COX-1 mRNA expression ($p > 0.05$). The COX-2 mRNA expressions were significantly different ($p < 0.05$), with the exception of that between infected cells with PTPBS and uninfected cells with PTPBS ($p > 0.05$). Bars show the means; the error bars show the mean + 1.0 standard deviation.

6 Discussion

6.1 Experiment conditions

The basic method in most experiments in this study was carried out using cell culture. When the effects of the selected drugs on *C. pneumoniae* infection are studied in cell cultures, *in vitro*, the following questions have to be answered: what kind of factors influence the propagation of *C. pneumonia*; and what kind of factors would interfere with the results of the experiments?

In order to efficiently culture, transport, store, and recover the chlamydiae, various factors have been discussed during in the past few decades to insure the high-yield propagation. Apart from the factors such as cell line (Kuo & Grayston 1990), sonication (Warford *et al.* 1985), culture media (Herbrink *et al.* 1991), culture temperature and pH values (Theunissen *et al.* 1992), the supplements or content of culture media have also been well discussed. A culture medium supplied with appropriate cycloheximide, amino acids and FCS can enhance the production and infectivity of organisms considerably (Harper *et al.* 2000, Maass & Dalhoff 1995, Theunissen *et al.* 1992).

In this study, the selected drugs were used to pre-treat chlamydial EBs or HL cells or were accompanied during inoculation and either chlamydial EBs or HL cells were washed after treatment or inoculation, which made the situations more complicated. Some factors were very important and they strongly affected the results of experiments. Here, the effects of temperature, time and FCS overlapped and interfered with one another. When pre-treating HL cells at +35 °C for 1 h, cells were normal in the culture medium containing FCS but completely detached in the PBS. At +4 °C for 1 h, there was no significant difference between PBS and culture medium containing FCS, but the cells in PBS could become round and detached if the time span was longer than 1 h. Although it has been proposed to grow *C. pneumoniae* in a serum-free medium (Maass *et al.* 1993), our results showed the importance of FCS for HL cells and that the HL cell can maintain their viability in a FCS-free medium only for a short time and at a low temperature. Animal cells need a natural medium to provide the physical conditions such as pH and osmotic pressure or a medium supplemented with some natural product such as serum. FCS provides the cultured HL cells with nutrition and osmotic pressure and makes the medium more natural. The normal osmotic pressure is related to temperature: the higher the temperature, the higher the osmotic pressure. Living cells can perform

complicated chemical reactions at comparatively low temperatures, but they are rapidly destroyed if the temperature is slightly over the optimal temperature at which they operate best (Paul 1970). Serum also provides cultured cells with factors important for attachment and spreading (Maurer 1986). Pre-treatment of chlamydial EBs in PBS caused the loss of the infectivity of the organisms up to 90%, no matter which temperature, +4 °C or +35 °C, was used when compared to the culture medium containing FCS. Treatment of chlamydial EBs or HL cells with PBS can result in the loss of most EBs and cells especially at higher temperatures. This proved that FCS is an indispensable substance for the survival of chlamydiae and it has the stabilizing effect on the extracellular survival of *C. pneumoniae* (Theunissen *et al.* 1992). For these reasons, the medium used in this study was supplemented with 7.5% FCS either for pre-treatment of HL cells and chlamydial EBs or for inoculation. To minimize the protein interference of FCS in the experiments, the controls used the medium containing FCS as well.

Chlamydial EBs can be lost during washing procedure if they are removed with supernatant. Therefore, washing may cause a false interpretation that the infectivity of chlamydiae was decreased in the culture. In order to exclude this effect, the chlamydial EBs without any drug treatment were washed and left as unwashed. Washing did not decrease the number of inclusions; on the contrary, it increased the inclusion counts about three fold in the cultures. This is most probably due to the fact that washing procedure disaggregated the chlamydial EBs. Chlamydial EBs tend to aggregate after entering the host cells to be inaccessible to antibodies (Majeed & Kihlstrom 1991). After harvesting and purification, they may still gather to form clumps.

Sonication could possibly disaggregate the EB clumps thus enhancing the infectivity of chlamydiae (Warford *et al.* 1985). In this study, a 15-s sonication of chlamydial EBs did not influence the inclusion counts in the following cultures. On the contrary, when the duration of sonication was extended to 10 min, the inclusion counts decreased 30–40%. This suggests that sonication has no benefit to disaggregate *C. pneumoniae* EBs and that extended sonication may even be harmful to the EBs.

6.2   Antichlamydial effects of drugs used for cardiovascular diseases

The results of this study showed that all the investigated drugs had antichlamydial effects to a certain extent but their modes and mechanisms to exert the effect varied.

6.2.1 Heparan sulfate-like glycosaminoglycans

Heparin is the most important member of GAGs. A few other members were also tested. Because heparan sulfate-like GAGs are used by chlamydiae as receptors to attach to host cells (Stephens *et al.* 2001), the GAGs were applied at the earliest infective stage: before and during inoculation. The results of this study demonstrated, as also found earlier, that when GAGs were applied during inoculation they inhibited the infectivity of both *C. pneumoniae* and *C. trachomatis* and the inhibition was dose-dependent (Taraktchoglou *et al.* 2001, Wuppermann *et al.* 2001). One exception was *C. trachomatis* L2: CSA could not inhibit its infectivity, although heparin could almost totally eliminate it. This revealed that the inhibitory effect of GAGs on the infectivity of chlamydiae was strain-dependent and differed between GAG members. The results from the pretreatment of chlamydial EBs with GAGs clearerly demonstrated this dependent relation between inhibitory effects of GAGs and the strains.

Some of the findings in this study on the inhibitory effect of heparan sulfate-like GAGs are different from those obtained in earlier studies. One of the most interesting findings is that the infectivity of both *C. pneumoniae* K7 and Parola strains could be inhibited not only by heparin but also by CSA, which has not been effective in earlier studies (Stephens *et al.* 2000, Wuppermann *et al.* 2001, Zhang & Stephens 1992). The results showed that CSA could significantly, with a rate of more than 60%, inhibit the infectivity of *C. pneumoniae* strains K7, Parola and *C. trachomatis* serovar E but there was no effect on *C. trachomatis* serovar L2. This is in accordance with the inhibitory effect of CSA on *C. trachomatis* serovar E (Taraktchoglou *et al.* 2001) but it contradicts the effect on a German *C. pneumoniae* strain GiD (Wuppermann *et al.* 2001) and the earlier results with *C. trachomatis* serovar L2 (Stephens *et al.* 2000, Zhang & Stephens 1992). It seems that the Finnish *C. pneumoniae* strains K7 and Parola are somewhat different from the German *C. pneumoniae* strain GiD and American *C. pneumoniae* strains CWL029 which were inhibited by about 37% in this study. It has been reported that

the ability of microbes binding to GAGs and using GAGs as ligands to mediate attachment to host cells is variable. For example, one *Borrelia burgdorferi* strain can bind to both heparan sulfate and dermatan sulfate, but another strain can bind perhaps only to dermatan sulfate (Parveen *et al.* 2003). Although CSA was not considered a ligand for chlamydiae (Stephens *et al.* 2000, Zhang & Stephens 1992), it has been shown to mediate the attachment of HSV to host cells (Mardberg *et al.* 2002). Inhibition of CSA on the infectivity of *C. pneumoniae* in this study varied from 37% to 65%, suggesting the GAG receptors on the outer surface of *C. pneumoniae* could be variable, i.e., the GAG receptors on the surface of some strains may be more specific to heparin or heparan sulfate while of other strains may be less specific, binding both heparin and CSA. There might be some differences between the receptors of the American strain CWL029 and Finnish isolates K7 and Parola.

Another interesting finding is that the pretreatment of chlamydial EBs with GAGs lost the inhibitory effects of some GAGs on some chlamydial strains. Different heparin preparations could more or less inhibit the infectivity of *C. pneumoniae* K7 and *C. trachomatis* L2 and E but were not effective against *C. pneumoniae* CWL029 and Parola strains. Both Parola and K7 are Finnish strains, but K7 was isolated in 1987 (Ekman *et al.* 1993) during a wide epidemic from a military conscript in northern Finland, whereas Parola was isolated some years later during a nonepidemic period from a military conscript in southern Finland. Particularly, CSA not only lost its inhibitory effect but enhanced the infectivity of *C. pneumoniae* CWL029 and Parola even over 200%. If the inhibitory effect of GAGs present during inoculation was nearly identical between *C. pneumoniae* strains used in this study, the pretreatment of chlamydial EBs with GAGs, especially with CSA, revealed the diversity of *C. pneumoniae* strains; the difference were seen between the American strain and Finnish strain, and even between Finnish strains. *C. pneumoniae* and *C. trachomatis* have been reported to possess only a few CSA molecules on their surface providing an opportunity for receptor saturation (Beswick *et al.* 2003). The enhancing effect of CSA seen in our experiments suggested that *C. pneumoniae* strains CWL029 and Parola, but not K7, might have CSA receptors on their outer surfaces which were saturated with CSA during pretreatment, thus enhancing the binding to receptors in the host cells. Quantitative real-time PCR, as the control experiment in this study, showed the relative dispersion and the viability relative to the total number of included chlamydial EBs. Its results suggested that one inclusion could be caused by more than one single EB

or the viability of EBs lost significantly during experiments; therefore, the possibility of chlamydial infectivity being enhanced to over 200% exists.

Previous studies have shown that pretreatment of host cells with GAGs is inhibitory to the infectivity of *C. pneumoniae* TW183 and A-03 (Beswick *et al.* 2003) but not to that of *C. pneumoniae* GiD (Wuppermann *et al.* 2001). All GAGs used in the present study effectively inhibited the infectivity of *C. pneumoniae* CWL029, K7 and Parola. One possible reason for the differences between these studies might be the use of different cell lines as host cells: HEp-2 was used for GiD, bronchial epithelial cells for TW183 and A-03, and HL cells for CWL029, K7 and Parola. On the other hand, one cannot exclude the possibility of diversity between *C. pneumoniae* strains although it has been suggested earlier that the omp2 gene is identical in all *C. pneumoniae* strains (Wagels *et al.* 1994). The omp2 gene encodes chlamydial 60000 molecular weight cysteine-rich outer membrane complex protein OmcB which functions as a ligand in the chlamydial attachment to host cells and serves as the acceptor molecule responsible for binding heparan sulfate like GAGs (Stephens *et al.* 2001). Chlamydial attachment to host cells is mediated by heparin, and the GAG receptors of the host cells and chlamydiae need different binding sequences (Chen *et al.* 1996). The results of this study suggest that exogenous heparan sulfate GAGs could compete with *C. pneumoniae* for host cell receptors and thus inhibit the infectivity of chlamydiae. The competitive ability of heparin depends on the structure of heparin (Chen *et al.* 1996), and the structure of the heparan sulfate GAGs produced by different host cells depends on the cellular origin (Gallagher & Walker 1985). This may partially explain the difference between the results in this study and other studies. There might also be some geographical differences between *C. pneumoniae* strains (Palys *et al.* 1997). The results in this study only presented the variations of pathogenicity between different *C. pneumoniae* strains *in vitro*, but the virulence diversity between different *C. pneumoniae* strains have also been revealed recently *in vivo* (Sommer *et al.* 2009) which were not recognized before. Clinically, heparins are used in the patients with acute coronary syndromes only during the first couple days, where at this time their antichlamydial effect may be effective only to the spread of newborn chlamydial EBs.

6.2.2 Selective COX inhibitors

The results in this study demonstrated that during 72 h incubation after inoculation of chlamydial EBs, the presence of selective COX inhibitors in the culture medium

could affect the growth of *C. pneumoniae*. In conjunction with the increase of the concentration of the drugs, selective COX inhibitors could inhibit the growth of *C. pneumoniae* from almost no effect when the concentration was under 1μg/ml to a total lack of the growth at the concentration 10 μg/ml SC-560 or 18 μg/ml PTPBS. By using confocal microscopy, smaller inclusions also confirmed the inhibitory effect of selective COX inhibitors.

However, the results from experiments on the recovery of *C. pneumoniae* after treatment showed that the inhibitory effects of selective COX inhibitors on the growth are reversible. By either extending the culture to an extra 72 h in a fresh culture medium without any COX inhibitors or repassaging infected cells to new cell monolayers and incubating them in a culture medium without COX inhibitors, chlamydial inclusions reappeared even in the cultures which had been completely inhibited in the first 72 h incubation or first passage with the highest drug concentrations. These findings suggest that chlamydiae are still viable even with the highest concentration of selective COX inhibitors and that the COX inhibitors could only inhibit or delay the development of chlamydiae but not kill them, i.e., they are chlamydiostatic but not chlamydiacidic.

The expression of COX-2 is induced by inflammation (Burleigh *et al.* 2002, O'Neill & Ford-Hutchinson 1993), and *C. pneumoniae* infection could cause a 10-fold rise in COX-2 mRNA expression in peripheral blood monocytes (Rupp *et al.* 2004). The quantitative analysis of COX-2 mRNA expression in this study indicates clearly that *C. pneumoniae* infection reduces the expression of COX-2 mRNA in HL cells. This difference in results is probably due to the different cell lines or different chlamydial strains used. As expected, the application of COX-2 inhibitor PTPBS also reduced the expression of COX-2 mRNA. Since both COX-2 inhibitor PTPBS and *C. pneumoniae* infection can downregulate the COX-2 mRNA expression, theoretically one would expect to see greater downregulation in *C. pneumoniae* infected cells treated with PTPBS. Interestingly, on the contrary, the enhanced downregulating effect was not seen in *C. pneumoniae* infected cells treated with PTPBS. We suppose that in *C. pneumoniae* infected cells treated with the COX-2 inhibitor, one could see only (or mostly) the downregulation of the COX-2 mRNA expression caused by the COX-2 inhibitor because the inhibitor suppressed the growth of *C. pneumonia*; thus it disabled the downregulating effect of chlamydiae. It is still unclear whether the downregulated COX-2 mRNA expression is the mechanism by which the COX-2 inhibitor PTPBS inhibits the growth of *C. pneumoniae* in HL cells. A study with *Chlamydia muridarum* reported that the maximal production of *C. muridarum* EBs COX-2 is needed. The

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inhibition of COX-2 by selective COX-2 inhibitors can influence the growth of *C. muridarum* (Liu *et al.* 2006). In the results of this study, COX-1 mRNA expression of HL cells was not significantly affected by either the infection of *C. pneumoniae* or the treatment of selective COX-1 inhibitor SC-560. This suggests that the inhibition of the growth of *C. pneumoniae* caused by SC-560 is irrelevant to COX-1 mRNA expression. In other words, there must be other mechanisms for SC-560 to inhibit the growth of *C. pneumoniae*.

Since chlamydiae are obligate intracellular bacteria, the metabolism and activity of host cells are very important to the survival and growth of chlamydiae. Any changes of the metabolism or activity of host cells would possibly affect the growth of chlamydiae. Except acting directly on microbes, it is also conceivable that selective COX inhibitors inhibit the growth of *C. pneumoniae* by indirectly affecting their host cells. In 1999, Dechend *et al.* reported that *C. pneumoniae* infection could activate nuclear factor κB (NF-κB) (Dechend *et al.* 1999) which may contribute to the life cycle of chlamydiae; and Tiran *et al.* showed that by restraining the *C. pneumoniae*-induced NF-κB activation, the unselective COX inhibitor, aspirin, can inhibit the development of *C. pneumoniae* in human endothelial cells (Tiran *et al.* 2002). From this, it can be speculated that the restraint of *C. pneumoniae*-induced NF-κB activation may be one of the mechanisms for selective COX inhibitors to inhibit the development of *C. pneumoniae*.

6.2.3 Rapamycin

The results in this study demonstrate that rapamycin possesses indubitable antichlamydial properties on *C. pneumoniae*. The pretreatment of host cells or *C. pneumoniae* EBs with rapamycin did not affect the growth of *C. pneumoniae* significantly which indicates that the inhibitory effect of rapamycin on the growth of *C. pneumoniae* is not exerted at the initial phase, attachment and entering into the host cells, but the succeeding developmental phases. This is concurrent with the property of rapamycin blocking cell cycle progression in G1 phase, thus inhibiting the cells from entering the S/G2/M phase (Gaben *et al.* 2004, Javier *et al.* 1997, Powell *et al.* 1999). From confocal and electron microscopy, the morphological changes of inclusions of *C. pneumoniae* under the influence of rapamycin could be seen. The presence of rapamycin during the life cycle of *C. pneumoniae*, particularly during the phase of growth, caused less inclusion counts, smaller inclusion size, less chlamydial particles in the inclusions, more non-infective RBs and elongated RBs. The presence of more non-infective RBs and elongated RBs

suggests that the replication of RBs by binary fission and the maturation from RBs to EBs were blocked by rapamycin and stopped in the G1 phase. The further decrease of inclusion counts after repassaging the infected cells grown in the medium containing 11 μg/ml of rapamycin also showed that the immature RBs might be the cause of reduced infectivity of the formed inclusions in the first passage.

Besides this possible mechanism of stopping replication and maturation of RB to inhibit the growth of *C. pneumoniae*, rapamycin may also affect *C. pneumoniae* infection by affecting their macrophage infectivity potentiator (MIP) proteins. MIPs are considered as virulence factors in intracellular pathogens. The MIP proteins in *C. trachomatis* and *Trypanosoma cruzi* were shown homologous to FK506-binding proteins (FKBPs, which exhibit peptidyl prolyl *cis-trans* isomerase (PPlase) activity) (Lundemose *et al.* 1993, Moro *et al.* 1995). Even with immunocytochemistry the surface-exposed MIP epitopes cannot be shown on the surface of either *C. trachomatis* EBs or RBs, the antibody-accessible MIP epitopes are suggested to be present on the surface of EBs because the antibodies against the N-terminal segment of MIP protein inhibited infectivity of *C. trachomatis* up to 90% (Lundemose *et al.* 1993). In *C. pneumoniae* and *C. psittaci*, the MIP proteins have been identified on the surface of both EBs and RBs (Montigiani *et al.* 2002, Rockey *et al.* 1996). It has been demonstrated the MIP homologue Cpn0661 of *C. pneumoniae* is a secreted effector protein providing the MIP protein with access into the host cell cytoplasm and can be demonstrated on the surface of inclusion from 20 to 72 hours post-infection (Herrmann *et al.* 2006). The presence of MIP protein in *C. pneumoniae* suggests that rapamycin, an immunosuppressant similar to FK506, may affect PPlase activity and thus interferes with the infection. In this study rapamycin reduced the development of both *C. trachomatis* and *C. pneumoniae* most clearly during the first 24 hours and, thus, it is supposed that the inhibition of the MIP isomerase in the early phase of infection plays an important role.

It has been shown that roxithromycin prevents restenosis in patients with high *C. pneumoniae* antibody titres (Neumann *et al.* 2001), and doxycycline does that in male smokers (Kannengiesser *et al.* 2004) who seem to be highly susceptible to chronic *C. pneumoniae* infection (Smieja *et al.* 2002, von Hertzen *et al.* 1998). Several recent studies also suggested that *C. pneumoniae* infection may worsen the prognosis in lung, liver and heart transplantation (Glanville 2001, Haubitz *et al.* 2004, Kotsimbos *et al.* 2005, Kwiatkowski *et al.* 2006, Lotz *et al.* 2004, Subramanian *et al.* 2002, Wittwer *et al.* 2000). *C. pneumoniae* has also been

implicated in chronic allograft rejection (Kwiatkowski *et al.* 2006). This microbe also accelerates graft arteriosclerosis after cardiac transplantation and is associated with graft rejection after lung transplantation. These findings hint that the beneficial effects of rapamycin in the prevention of in-stent restenosis, anti-atherosclerosis and on transplantation patients are at least partly due to its antichlamydial effect. However, the rapamycin levels found in human sera during clinical standard treatment of transplant patients are only 3.0–18.0 ng/ml which is 1000 times lower than the optimal concentrations for inhibiting the development of *C. pneumoniae*, *in vitro*, in this study. Therefore, the most clinical outcomes of rapamycin in the treatment of allograft rejection should be contributed by merely the immunosuppressive activity but not the antichlamydial activity. Wessely *et al.* reported a rapamycin eluting stent platform in which maximum tissue levels in the stented coronary vascular wall were more than 20 ng/mg (Wessely *et al.* 2005). Levy *et al.* also reported the tissue concentration of canine cerebral vascular tissues near the rapamycin eluting-stent reached around 50 ng/mg (Levy *et al.* 2006). These concentrations achieved are 2-fold higher than the optimal concentration for inhibiting the development of *C. pneumoniae* in the present study. Therefore, the findings in this study suggest that the beneficial effects of rapamycin in the prevention of in-stent restenosis might partly be explained by its antichlamydial effect, especially the effect on the new infection caused by newborn chlamydial EBs.

Other drug

In addition to abovementioned heparan sulfate-like GAGs, selective COX inhibitors and rapamycin, our unpublished results have also demonstrated that verapamil can inhibit the infectivity of *C. pneumoniae in vitro*, the effective concentration of verapamil depending on the application methods and chlamydial strains: when HL cells were pre-treated with verapamil, the inhibitory concentrations to *C. pneumoniae* K7 were 200 to 1000 μM, to *C. trachomatis* L2 were 500 to 1000 μM. Pre-treatment of chlamydial EBs with 50 to 1000 μM of verapamil inhibited 99% of infectivity of *C. trachomatis* L2 but 80% of that of *C. pneumoniae* K7. It seems that *C. pneumoniae* is less susceptible to blocked Ca^{2+} channel of HL cells than *C. trachomatis*. Verapamil was present through the whole incubation period (48 h for *C. trachomatis*, 72 h for *C. pneumoniae* K7), and the effective concentrations were 50 to 400 μM. It suggests that the longer time the drug was applied, the lower concentration was required for the effect. The reason

might be that the persistent presence of verapamil affected the aggregation of chlamydial EBs in infected cells and the aggregation of intracellular EBs may prevent themselves from fusion with host cell lysosomes, and thus, the differentiation of RBs into EBs (Majeed *et al.* 1993, Shainkin-Kestenbaum *et al.* 1989). Chlamydial EBs become enclosed in membrane-bound endosomes and aggregate intracellularly after entering the eukaryotic cells and the formation of chlamydial aggregates and inclusions require a physiological concentration of intracellular ionized calcium ($[Ca^{2+}]_i$) (Majeed *et al.* 1993). Therefore if calcium fluxes were blocked, the growth of chlamydiae must be interrupted. In addition, verapamil may inhibit the intracellular calcium release caused by the contact of chlamydiae with HL cells, thus preventing the chlamydiae-induced changes on the F-actin cytoskeleton and NF-κB activation (Wissel *et al.* 2005). Verapamil may improve *C. pneumoniae* susceptibility to antibiotics by blocking the L-type Ca^{2+} channel (Azenabor *et al.* 2003) in spite of its own anti-viral and antichlamydial property. The benefits of verapamil also include down-regulating lipoprotein lipase (LpL) gene expression in *C. pneumoniae*-infected macrophages. LpL gene expression may be upregulated in a manner that favors atherogenic properties by Ca^{2+} signalling in *C. pneumoniae*-infected macrophages (Azenabor *et al.* 2004). Since atherosclerosis is associated with chronic *C. pneumoniae* infection, the antichlamydial property of the drugs used in cardiovascular diseases may play a role in its anti-atherosclerosis effect. Of course, the clinical application of these drugs on antichlamydial infections needs further studies.

7 Summary and conclusions

Cell culture is a useful method in studying *Chlamydia in vitro*. Almost any conceivable factor may, however, affect the results of experiments. This study showed the complex interaction between temperature, time and media during the pre-treatment before inoculation. These factors not only can influence the results separately, but their influences also overlap and interlace each other. This study also showed that the simple washing procedure could enhance the infectivity of *C. pneumonia*, although this procedure generally is considered to cause loss of chlamydial EBs and sequentially decrease the infectivity of chlamydiae. Therefore, once an experiment model is established, it is necessary to test every experimental condition, special procedure or medium to check how much these factors can affect the results. To assure the reliable results, any possible factors which may influence the experiments should be acknowledged.

Because of the association between the chronic *C. pneumoniae* infection and CVD and atherosclerosis, the drugs used in the treatment of cardiovascular diseases were tested for their antichlamydial activity. The results of this study show that all investigated drugs possess, to different extents, antichlamydial activity which might be more or less beneficial on their clinical efficiency in the treatment of cardiovascular diseases. Although the detailed mechanisms by which the investigated drugs inhibit the infectivity of *C. pneumoniae* was not studied here, it is obvious that selective COX inhibitors, rapamycin and verapamil can inhibit the infectivity of *C. pneumoniae* by inhibiting the growth and maturation of *C. pneumonia*; whereas heparan sulfate-like glycosaminoglycans do this maybe by inhibiting the attachment of *C. pneumoniae* EBs onto the host cells. The recovery and repassage results of *C. pneumoniae* EBs after treatment in this study show that the growth can be only delayed by selective COX inhibitors and can recover to normal growth once the drugs are removed. However, rapamycin inhibits the infectivity of *C. pneumoniae* by inhibiting the maturation of chlamydial EBs, and therefore the infectivity falls down further even when the rapamycin was removed. This also indicates the necessity of the continuous use of drugs such as aspirin and selective COX inhibitors to control infection of chlamydiae.

An important aspect that needs to be kept in mind is the dose and the application route of the drugs. Used in organ transplantation to minimize the postoperative complications and to maintain the survival of the allograft in the patient, rapamycin is systemically administrated. The dose needed to keep the serum concentration is around 3–18 ng/ml, which is far lower than the effective

antichlamydia concentration in this study. When used in drug-eluting stent to inhibit the neointima formation and minimize the restenosis, rapamycin is locally applied and the local tissue concentration of rapamycin can achieve concentrations which are even 2 folds over the effective antichlamydia concentrations. Furthermore, this present study also supplied evidence for, at least *in vitro*, the existence of the pathogenicity diversity between *C. pneumonia* strains.

This study is based on *in vitro* experiments which were the model of the acute infection, although most important clinical infections caused by *C. pneumoniae* are chronic. Therefore the drugs studied here seem to be helpful in preventing new spread of chlamydial EBs and in inhibiting new infections, as LMWHs used only in patients with acute coronary syndromes, rather than to treat the chronic chlamydial infections. The clinical applications of the antichlamydial properties of these drugs need to be further studied. Further studies could also include whether there are new chlamydial EBs released in the patients with chronic chlamydial infection and the investigation of the antichlamydial effects of other drugs used in cardiovascular diseases, such as ARBs.

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I   Yan Y, Silvennoinen-Kassinen S, Leinonen M & Saikku P (2005) Methodological aspects affecting the infectivity of *Chlamydia pneumoniae* in cell cultures *in vitro*. J Microbiol Methods 61: 127–130.

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1015. Girsén, Anna (2009) Preeclampsia and maternal type-1 diabetes: new insights into maternal and fetal pathophysiology

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