The potential role of *Chlamydia pneumoniae* infection in the progression of atherosclerosis

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TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

Abstract

The potential role of *Chlamydia pneumoniae* infection in the progression of atherosclerosis

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Background: Chronic *Chlamydia pneumoniae* infection has been implicated as important etiologic factor of atherosclerosis, in particular coronary artery disease (CAD). Some experimental evidence links *C pneumoniae* with the induction of matrix metalloproteinases (MMPs). However, the regulation of MMPs by *C pneumoniae* within atheroma remains unclear. A recent study suggested that an EMMPRIN/MT1-MMP system exists that induces and activates MMP, and that is upregulated in the failing human left ventricle. In the present study, we examined the seropositive rate of *C pneumoniae* antibody in CAD patients, and investigated the relationship between *C pneumoniae* and the expression of MMPs in human atherosclerotic specimens. Furthermore, we investigated an EMMPRIN/MT1-MMP system that induces/activates MMPs in *C pneumoniae* infected atherosclerotic tissues and examined its potential role in the progression of atherosclerosis.

Materials and methods: We tested the prevalence of *C pneumoniae*

infection in CAD patients (n=391) and controls (n=97) by serologic study. In the present study, we evaluated the relationship between *C pneumoniae* and atherosclerosis by using a seroepidemiologic study. In addition, we performed histopathological and in vitro analysis in human atherosclerotic aortic and carotid artery tissues obtained from patients seropositive to *C pneumoniae* (n=20), by using antibodies to *C pneumoniae*, EMMPRIN/MT1-MMP, MMP-2, MMP-9, COX-2 and TIMP-2.

Results: The seropositive rates of both anti-*C pneumoniae* IgG and IgA in the CAD patients were elevated in subgroups without conventional coronary risk factors compared with subgroups with conventional risk factors. Immunoreactivities of EMMPRIN, MT1-MMP, COX-2, MMP-2, and MMP-9 were increased in the atherosclerotic plaque itself, predominantly in immunoreactive macrophages/foam cells to *C pneumoniae*. Furthermore MMP-2 and MMP-9 zymographic activities were more increased in *C pneumoniae* infected atheromatous tissues compared with control tissues. Western blot for EMMPRIN, MMP-2 and TIMP-2 demonstrated that EMMPRIN, MMP-2 and TIMP-2 were detected more prominent in *C pneumoniae* infected atheromatous tissues compared with control tissues.

Conclusion: These findings help to understand the potential role of *C pneumo[1)]niae* in the progression of atherosclerosis.

Key Words: atherosclerosis, *Chlamydia pneumoniae*, EMMPRIN, MMPs,

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I. Introduction

Several classic risk factors have been identified for the development and progression of atherosclerosis. and coronary artery disease (CAD). However, these factors may explain only the high prevalence of CAD in part[1].

Injury to a vessel wall and the associated inflammatory response are now generally recognized as essential components of atherogenesis. However, the stimuli that initiate and sustain the inflammatory process have not been fully identified. Infectious insult is a candidate trigger of immuno-inflammatory response, and might be a source of chronic local or systemic inflammation[2].

Recently, substantial seroepidemiologic and some experimental evidence has linked *Chlamydia pneumoniae* (*C pneumoniae*) with the natural history of atherosclerosis[3,4]. However, the mechanisms by which this agent affects atherogenesis and the progression of atherosclerosis remain poorly understood. *C pneumoniae* is an obligate, intracellular, Gram-negative bacterium and airborne infection with this agent, often chronic and asymptomatic, is prevalent

in the general population. Moreover, an antibody titer against *C pneumoniae*, detectable in 40% to 50% of the adult population, has been found to correlate positively with the occurrence of coronary artery disease[5,6]. In the growth cycle of *C pneumoniae* there are 2 morphologically and functionally distinct cell types: the infectious elementary body (EB) and the reproductive reticulate body (RB)[7]. Under some conditions, certain chlamydia can achieve a state of intracellular chronic, persistent infection in which they remain viable but metabolically quiescent and do not replicate. During such chronic, persistent infection *C pneumoniae* expresses basal levels of two major antigens: the major outer membrane protein (MOMP) and the heat shock protein 60 (HSP 60; 60 stands for 60kDa). Although *C pneumoniae* can infect most cells present in atheroma[8,9,10,11], it localizes mainly to macrophages/monocytes in the plaque. These lesional macrophages/monocytes can produce matrix metalloproteinases (MMPs)[12], enzymes now accorded a major role in the degradation of the extracellular matrix of vascular tissue[13]. Thus, macrophage/monocytes-derived MMPs may play a role in the vulnerability of plaque and consequent thrombosis, and ultimately the progression of atherosclerosis and the acute coronary events[14].

MMPs are a family of enzymes that, as a group, can degrade a wide variety of extracellular matrix components and have been implicated in normal tissue remodeling as well as in inflammatory processes, tumor invasion, and wound healing. Seventeen MMPs are expressed in vitro by a variety of vascular cells, including macrophages, smooth muscle cells, and endothelial cells[15,16,17]. Recently, Kol at al.[18] reported that chlamydial HSP 60 derived from *C trachomatis* stimulate the expression of tumor necrosis factor-α (TNF-α) and MMP-9 by mouse peritoneal macrophages and Kreula et al.[19] reported that *C pneumoniae* proteins induce the secretion of the 92-kDa gelatinase (MMP-9) by human monocyte derived macrophages. However, it remains unclear

whether MMPs are regulated by *C pneumonia* within atheromatous plaques and whether the upstream cellular and molecular mechanisms of local MMP induction/activation system exist associated with *C pneumoniae.*

Recently, a tumor-derived protein, extracellular matrix metalloproteinase inducer (EMMPRIN), was found to induce the production of MMPs from stromal fibroblasts and to be crucial in tumor invasion[20]. In addition, it has been reported that MMP-9 and EMMPRIN were increased in the LV myocardium of ischemic DCM(dilated cardiomyopathy) and nonischemic DCM[21]. Moreover Major et al.[22] reported that EMMPRIN is induced upon monocyte differentiation and is expressed in human atheroma. EMMPRIN is a 58-kDa, membrane-bound protein that has been identified in both normal and diseased human tissue. It also known as basign or CD147, and is a glycoprotein, which is enriched on the surface of tumor cells, and which stimulates the production of several matrix metalloproteinases by adjacent stromal cells. The exposure of human fibroblasts to recombinant EMMPRIN causes the induction of MMP-1, MMP-2 and MMP-3, and basal expression of EMMPRIN has been reported in a number of tissue types, suggesting that this transmembrane protein has multiple roles[23].

In the present study, we evaluated the relationship between *C pneumoniae* and atherosclerosis seroepidemiologically. To investigate the potential role of *C pneumoniae* in the progression of atherosclerosis, we performed histopathological and in vitro analyses in human atherosclerotic aortic and carotid artery tissues, obtained from patients who were found seropositive for *C pneumoniae*, by using antibodies to *C pneumoniae*, EMMPRIN/MT1-MMP, MMP-2, TIMP-2 and MMP-9. The presence of the organism in atherosclerotic plaques has been investigated using various techniques such as immunohistochemistry (IHC), polymerase chain reaction (PCR), electron microscopy and isolation in tissue culture, but generally, IHC provides better

evidence for *C pneumoniae* than PCR in that point it can directly show the protein expression and colocalization. Therefore, we tested immunoreactivity for *C pneumoniae* and inflammatory mediators, such as, MMP-2, and MMP-9, to determine whether they are colocalized to inflammatory cells in atheromatous plaque, and to determine if *C pneumoniae* has some pathogenic role on atherosclerotic disease. That is, if the vascular infection of *C pneumoniae* can induce a chronic inflammatory reaction in host vascular tissue and activate inflammatory cells, some inflammatory mediators, such MMP-2 and MMP-9 may show increased expression around macrophages, which have a key role in atherosclerosis, infected with *C pneumoniae.* Furthermore, IHC of the EMMPRIN/MT1-MMP system, the upstream cellular and molecular mechanisms for a local MMP induction/activation system, was performed to investigate the potential role of *C pneumoniae* in the progression of atherosclerosis.

II. Materials and Methods

1. Seroepidemiologic study

Among the patients with typical symptoms of angina and with positive results in non-invasive testing (EKG, Treadmill test) who visited Yong-Dong Severance Hospital, 391 patients who underwent coronary angiogram were included in this study. Among them, the patients who demonstrated luminal narrowing of more than 50% in at least one vessel were grouped into the disease group (Group I, n=254) and those patients who had normal coronary arteries or minimal lesion were grouped into the positive control group (Group II, n=137). We also studied healthy persons who had not experienced any symptoms related to coronary heart disease and had normal findings on noninvasive tests for coronary artery disease and these subjects were grouped into the negative control group (Group III, n=97). Serologic tests for anti-chlamydial Ig G and Ig A were performed using ELISA kit (Bioclonic Inc., Sydney, Australia).

2. Tissue preparation and histologic examination

The study population consisted of 20 patients with atherosclerotic aortic (5 patients) and carotid artery diseases (15 patients). All patients were seropositive to *C pneumoniae* either with Ig G or Ig A antibodies. Serologic test for anti-chlamydial Ig G and Ig A were performed using ELISA kit. For control studies, aorta specimens, from which atherosclerotic lesions including

fatty streak and plaque were excluded, were obtained from 5 patients who were surgically treated for traumatic aortic dissection with C *pneumoniae* seronegativity.

Immediately following the removal of the specimens, each was fixed with buffered 10% formalin in order to maintain vascular morphologic integrity. To preserve the integrity of the advantitia and perivascular tissues, the specimens were carefully removed in a segment along with adjacent tissues and rinsed with PBS (phosphate buffered saline). Each segment was embeded in paraffin and cut in 5 ㎛ sections, which were then stained with hematoxylin-eosin (H&E). Sections of these tissues were also used for the immunohistochemical staining procedure.

3. Immunohistochemical staining

Mouse anti-*C pneumoniae* monoclonal antibody (RR-402) (DAKO Inc., Carpenteria, CA, USA) and goat polyclonal antibodies against human MMP-2, MMP-9, COX-2 (cyclooxygenase-2), TIMP-2, MT1-MMP and EMMPRIN (Santa-Cruz Biothchnology Inc., Santa Cruz, CA, USA) were used as the primary antibodies for immunohistochemical staining. To characterize the type of infected cells, HAM56 (monoclonal mouse anti-human macrophage) was used for the immunostaining of the tissue type of macrophage/mononuclear cell. Anti-chlamydial antibody reacts with a major outer membrane protein (MOMP) of *C pneumoniae* and the immunogen is *C pneumoniae* strain TW183. Peroxidase-conjugated secondary antibodies were used with these primary antibodies.

The paraffin sections were deparaffinized and rehydrated and then the

sections are boiled with citric acid for 5 minutes in order to suppress nonspecific binding of the antibodies and to increase the exposure of antigens, and cooled at room temperature for 20 minutes. The sections were then treated with 0.3% H_2O_2 for 5 minutes to suppress endogenous peroxidase activity. After treatment with PBS (pH=7.2-7.4) for 5 minutes and application of 1:5 diluted anti-chlamydial primary antibodies (RR-402) and 1:100 diluted EMMPRIN, MT1-MMP, MMP-2, TIMP-2, MMP-9, COX-2 and HAM56 primary anribodies, the sections were incubated in a moist chamber for 1 hour. After washing and bathing for 5 minutes by PBS, the biotinylated secondary antisera cocktail including goat anti-mouse and anti-rabbit IgG diluted 1:400 was incubated on the slides for 15 minutes at room temperature in a moist chamber. The sections were then processed by the streptavidin-biotin-peroxidase complex method by use of the LSAB(+) kit (DAKO Inc. Carpenteria, CA, USA) and DAB solution (Research Genetics Inc., Huntsville, AL. USA) in order to produce a brown color at the site of reactivity. The sections were then counterstained with Mayer's hematoxylin.

4. Western blot

A 50㎍ proteins were subjected to 8-12% gradient SDS-PAGE gel and transferred to immunobilon-P membrane(Millipore, Bedford, MA, USA) at 12V for 1h. The membrane was blocked in 5% nonfat dry milk in TBST at 25° C for 1h. Proteins were detected using EMMPRIN, MMP-2, TIMP-2: 5 ㎍/mL and secondary antibody (human rabbit/mouse IgG, horseradish peroxidase-conjugated, Amersham) was used at 1:2000 dilution. Signals were detected with an ECL kit (Amersham), and exposed to X-ray film (Kodak,

Rochester, NY, USA).

5. Gelatine zymography

Enzymatic activities of MMP-2 and MMP-9 were investigated using **zymographic analysis**. The protein content of the cultured medium was by the method of Bradford, using serum albumin as standard. 50μg proteins of culture medium from HUVECs and CASMCs with leptin treatment (10mg to 40 mg) and control were mixed with 5 x sample buffer and loaded on a 11% SDS-PAGE gel containing with 0.1% gelatin for electrophoresis under 4° C cold room. Gels were reacted with collagenase buffer for 16h at 37° C, stained with 0.25% coomassie brilliant blue, and destained with 30% isopropanol in 10% acetic acid to visualize the MMP bands.

6. Statistical analysis

We used SPSSWIN 8.0 software for the statistical analysis and the seropositive rate of each group was compared by Chi-square test for univariate analysis and logistic regression for multivariate analysis. $P<0.05$ was regarded as a statistically significant difference.

III. Results

1. Seroepidemiologic study

A total of 488 persons were included in this seroepidemiologic study for anti-*Chlamydia pneumoniae* Ig G and Ig A (group I: 254 in the disease, group II: 137 in the positive control, group III: 97 in the negative control). The simultaneous seropositive rates of both Ig G and Ig A were 56.7%, 61.3%, and 43.3% in Group I, II and III, respectively, and there was a significant difference between group I and III (p=0.033, OR=1.71 (95% CI; 1.07-2.75)). In subgrouping by conventional risk factors, the seropositive rates of both IgG and IgA in group I and group III, respectively, were 52.1% and 31.0% (p=0.035, OR=2.4) in females, 54.4% and 31.4% (p=0.008, OR=2.6) in non-smokers, 61.5% and 42.7% (p=0.013, OR=2.1) in patients with normal blood pressure, 59.9% and 40.2% (p=0.004, OR=2.2) in non-diabetes, 58.6% and 41.5% (p=0.016, OR=2.2) in patients with normal cholesterol level (<240 mg/dl), 58.1% and 42.0% (p=0.038, OR=1.9) in patients with high HDL-cholesterol level (≥35 mg/dl), and 58.3% and 40.7% (p=0.018, OR=2.0) in patients with low LDL-cholesterol level (<160 mg/dl). By multivariate analysis using Logistic regression, a statistical significance was noticed in females (p=0.012, OR=5.2 (95% CI; 1.4-18.6)) and non-smokers (p=0.012, OR=3.9 (95% CI; 1.3-11.0)) (Tables 1 and 2).

2. Histopathologic analysis

The sections of aorta taken from traumatic dissection as the normal control showed no histological evidence of atherosclerosis, except for minimal intimal thickening, and showed normal patterns of elastic media, and no immunoreactivity to *C pneumoniae* and trace immunoreactivities for MMP-2, MMP-9, TIMP-2 and EMMPRIN in the minimal thickened intima, and no immunoreactivity in the media (Fig.1).

In contrast to the control group, the 20 case-specimens showed a thickened intima from necrosis and a lipid-laden plaque formation that characterized atherosclerotic aortas and carotid arteries. There was also a prominent inflammatory infiltration with mononuclear and foam cells in the atheromatous plaques. *C pneumoniae* was stained dark brown within the macrophages/mononuclear cells in 12 of 20 atheromatous tissues, mainly atheromatous plaques (Fig.2). In the atherosclerotic lesions, immunoreactivity for MMP-2, MMP-9, COX-2, EMMPRIN and MT1-MMP were evident in all cases along with plaques, primarily in macrophages/foam cells, intimal and medial smooth muscle cells, and endothelial cells of the intima. Within the intima, increased MMP-2, MMP-9, TIMP-2, EMMPRIN and MT1-MMP immunoreactivities were found to have a similar pattern and distribution within the area stained by *C pneumoniae* (Fig.3).

3. Western blot

Because EMMPRIN, MMPs, and TIMP share considerable homology, western blotting was performed to define the expression of these proteins

quantitatively and exclude cross reactivity by immunohistochemical staining. EMMPRIN, MMP-2 and TIMP-2 were more prominently detected in *C pneumoniae* infected atheromatous specimens than in control specimens (Fig. 4).

4. Gelatin zymography

In *C pneumoniae* infected atheromatous specimens, a 92-kDa band corresponding to the activity of MMP-9 and 72-kDa band corresponding to the activity of MMP-2 demonstrated prominent comparing with weakly constitutive activity of MMP-2 and non-induced activity of MMP-9 in control specimens quantitatively (Fig.5). Futhermore, gelatinolytic activity of MMP-2 was more prominent than that of MMP-9 in *C pneumoniae* infected atheromatous tissue demonstrated similar patterns in western blot for EMMPRIN, MMP-2/TIMP-2, which are the upstream cellular and molecular mechanisms for a local MMP induction/activation system.

IV. Discussion

Although it is tempting to consider *C pneumoniae* infection as a possible primary cause of atherosclerotic lesion formation in some cases, the data currently available do not justify this conclusion. Infection of the vascular wall with *C pneumoniae* is generally focal and does not affect all lesions examined, raising legitimate questions about the specificity and the biological significance of the detection of this agent within atheroma. However, a recent autopsy study showed greater frequency of chlamydial antigens in the cardiovascular tissues of patients who had died of ischemic heart disease than in those who died of noncardiac causes (64% versus 38%). Moreover, the effects of a focal infection might influence the pathobiology of the surrounding atherosclerotic environment[24]. The present seroepidemiologic results demonstrate a positive correlation between *C pneumoniae* infection and ischemic heart disease, particularly in subgroups with few conventional coronary risk factors. Therefore, we can ascertain that *C pneumoniae* may play a pathologic role in atherosclerosis in those with low risk. Furthermore, histopathological analysis demonstrated an abundance of *C pneumoniae* in macrophages/monocytes of the atheromatous plaques of aortas and carotid arteries obtained from cases who were defined as having seropositive *C pneumoniae.*

Many other seroepidemiologic and histologic results support an association between *C pneumoniae* infection and atherosclerosis, but the pathogenic mechanism involved is not yet clear. Kreula et al. reported that chlamydial heat shock protein 60 induces the secretion of 92-kDa gelatinase (MMP-9) by human monocyte derived macrophages, and that *C pneumoniae,* when present in a macrophage-containing inflammatory environment, actively participate in the destruction of the extracellular matrix[19]. Kol et al. also suggested that

chlamydia induce the macrophage production of TNF-α and MMPs[18].

In the present study, most of the cases showed increased expressions of MMP-2, MMP-9 and COX-2 where the immunoreactivity of *C pneumoniae* was abundant. Immunoreactivity for MT1-MMP and TIMP-2 were found to have distributions similar to those of MMP-2 and MMP-9. In addition, gelatine zymography showed increased MMP-2 and MMP-9 activities in infected atheromatous tissues, this was more prominent for MMP-2. In spite of the possibility of an innocent bystander effect, these findings suggest that *C pneumoniae* might increase the capacity of macrophages to produce gelatinase (MMP-2 and MMP-9) by activating the expression of this enzyme. Such an increase in the expression of gelatinase could result in increased proteolytic activity in the microenvironment of the stimulated macrophages, and lead to enhance extracellular remodeling. However, these results may contain somewhat limitations, for example, the present study did not include in vitro studies with a pure infected monocytes/macrophage cell line, but rather to use inhomogenous atheromatous human tissues, and therfore many factors other than *C pneumoniae* infection, could have affected our results. However, despite these limitations, our results suggested that not only MMP-9 (92-kDa gelatinase) but also, MMP-2 (72-kDa gelatinase) might play an important role in the extracellular remodeling of *C pneumoniae* infected atheromatous tissues.

MMP-2 and MMP-9 are commonly called gelatinases because of their high affinity for this substrate. Production of gelatinases by macrophages/monocytes has recently been observed in atherosclerotic aortic aneurysms, implying a potentially important role for these enzymes in atherosclerotic disease. COX-2 is another enzyme regulated by NF-*k*B, is responsible for the increased production of prostaglandins and thromboxane in inflammatory disease. The induction of COX-2 in monocyte and the resulting production of prostaglandin E_2 have been shown to be involved in the signal transduction pathway leading

to MMPs production in those cells. A possible pathophysiologic mechanism explaining these results is that the promoter region of the gene for gelatinase contains NF-*k*B binding sites, which play a critical role in the expression of this gene, therefore, the *C pneumoniae*-mediated effect on gelatinase (MMP-2, MMP-9) expression may be due to the activation of NF-*k*B. This possibility is supported by a recent observation that *C pneumoniae* infection of vascular smooth muscle and endothelial cells activates NF-*k*B[25]. The factors regulating the production of these MMPs in atherosclerotic lesions are not yet known. However, it is known that the activity of MMPs on substrates of the extracellular matrix depends on a balance between these enzymes and their endogenous inhibitors, the TIMPs. Since TIMP expression can be regulated by biological agents such as cytokines, we tested TIMP-2 expression on infected atheromatous tissue. Although TIMP-2 expression was observed by IHC of infected atheromatous tissue, its level was insignificant compared with other MMPs, and even its stimulated expression level was not high enough to achieve the 1:1 molar ratio required for full inhibition of gelatinase.

However, it remains unclear whether the upstream cellular and molecular mechanism of local MMP induction/activation system actually exists. Recently, Spinale et al.[21] demonstrated that MMP induction/activation system (EMMPRIN and MT1-MMP) exists in the human left ventricular myocardium and that this is upregulated in heart failure. Moreover Major et al.[22] reported that EMMPRIN is induced on differentiating monocytes and is expressed in human atheroma. They suggested: 1) monocyte to macrophage differentiation induces both EMMPRIN and MMP expression;, and that 2) EMMPRIN may play a role in atherosclerotic lesion formation and that the influx/differentiation of monocyte may be responsible for potentially destabilizing atheroma. Through immunoprecipitation experiments, Berditchevski and colleagues[26] demonstrated that EMMPRIN forms a complex with a3b1 integrin. The functions of integrins

include cell-cell adhesion, extracellular matrix-cell adhesion, and the transduction of cellular signaling cascades. The coexistence of EMMPRIN and a3b1 integrin suggests that EMMPRIN mediated MMP induction may be influenced by both the composition and the level of stress placed on the extracellular matrix. The intracellular signaling pathways by which EMMPRIN facilitates MMP expression remain to be fully elucidated, but probably involve tyrosine kinase pathways. The EMMPRIN protein sequence contains a PKC phosphorylation site, which may also be an important intracellular regulatory mechanism[27]. In vitro studies have demonstrated that although EMMPRIN induces MMP expression, it does not influence the basal expression of TIMP[28].

In the present study, a similar pattern of expression was observed in infected atheromatous tissue, in which increased EMMPRIN expression was associated with the increased expressions of MMP-2, and MMP-9, and minimal TIMP-2 expression. Thus, increased EMMPRIN expression in infected atheromatous tissues may contribute to increased MMP levels without a concomitant increase in TIMP expression, which in turn would ultimately favor matrix degradation and remodeling. This provides circumstantial evidence that EMMPRIN may facilitate MMP-2 and MMP-9 expression in infected atherosclerotic plaques.

In conclusion, in this study we found that *C pneumoniae* may be an independent risk factor of atherosclerosis. Moreover, *C pneumoniae* was abundant and colocalized with MMP-9, MMP-2 and EMMPRIN in the atheromatous plaques of infected patients. Despite the possibility of innocent bystander effect, this finding suggests that *C pneumoniae* may play an important role in atherosclerosis and that a possible mechanism involve infected macrophage differentiation and the possible induction of MMP-2, and MMP-9 and the expression of EMMPRIN, and further that MMP-2 and MMP-9 participate in the degradation of the extracellular matrix component.

This study helps us to understand the molecular pathways by which allow *C pneumoniae* to participate in atherogenesis and to explain the mechanisms of the epidemiologic and pharmacologic links between this infectious agent and the clinical manifestations of atherosclerosis. Future studies involving EMMPRIN mediated MMP upstream regulation are necessary to fully elucidate the potential role of EMMPRIN in the progression of atherosclerosis.

V. Conclusion

The present study demonstrated that *C pneumoniae* may be an independent risk factor of atherosclerosis. Moreover, *C pneumoniae* was found to be abundant and to be colocalized with MMP-9, MMP-2 and EMMPRIN in the atheromatous plaques of infected patients. In spite of possibility of an innocent bystander effect, this finding suggests that *C pneumoniae* plays an important role in atherosclerosis and that mechanism involves infected macrophage differentiation that induces MMP-2, MMP-9, COX-2 and expresses EMMPRIN, and that further MMP-2 and MMP-9 participate in the degradation of the extracellular matrix component.

We believe that the present study helps in the understanding of pathobiological effects by which *C pneumoniae* might participate in atherogenesis and to explain the mechanism of the epidemiologic and pharmacologic links between this infectious agent and the clinical manifestations of atherosclerosis. Future study involving EMMPRIN mediated MMP upstream regulation is necessary to fully elucidate the potential role of EMMPRIN in the progression of atherosclerosis.

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국문 요약

동맥경화의 발생 및 진행에 있어 만성 *Chlamydia pneumoniae* 감염의 역할

배경: 만성 *Chlamydia pneumoniae* 감염은 관상동맥 질환을 포함한 동맥경화의 발생 및 진행의 중요한 위험인자임이 제시되고 있으며, 이러한 기전이 matrix metalloproteinase (MMP)를 통해 이루어 진다는 여러 연구가 보고되고 있다. 그러나 *Chlamydia pneumoniae*감염이 MMP의 발현과 활동을 조절하는 정확한 기전은 밝혀져 있지 않으며, 단지 감염된 대식세포에서 분비되는 cytokine등 여러인자가 작용한다고 알려져 있을 뿐이다. 최근 이러한 MMP를 유도하고 활성화 시키는 조절인자인 EMMPRIN/MT1-MMP체계가 심근 및 동맥경화조직에서 발견되어 본 실험에서는 혈청학적검사와 동맥경화 조직을 이용한 MMP, EMMPRIN/MT1-MMP의 면역검사를 통하여 *C pneumoniae* 감염이 동맥경화를 진행시키는 기전을 알아보고자 하였다.

대상 및 방법: 관상동맥질환을 가지고 있는 391명의 환자와 정상대조군 97명을 대상으로 *C pneumoniae*감염의 혈청학적 양성률을 측정하였으며, 혈청학적으로 *C pneumoniae* 항체 양성이며 동맥경화성 대동맥류와 경동맥 협착증이 있는 20명의 환자에서 얻은 동맥경화 조직을 대상으로 *C pneumoniae*, MMP-2, MMP-9, TIMP-2, EMMPRIN/MT1-MMP 의 면역형광염색과 western blot, gelatine zymography를 시행하였다.

결과:관상동맥질환을 가진 환자에서 대조군보다 높은 *C pneumoniae* 항체 양성률을 보였으며, 특히 일반적인 동맥경화의 위험인자를 가지지 않은 군에서 위험인자를 가진군보다 항체 양성률이 높았다. 또한 20명의 동맥경화조직중 12명의 조직에서 *C pneumoniae*를 확인할수 있었으며, *C pneumoniae*가 존재하는 대식세포 주위로 MMP-2, MMP-9, EMMPRIN의 표현이 증가되어 있으며, 정상대조군에 비해 MMP-2 와 MMP-9의 활성화도 증가되어 있는 것을 확인 할 수 있었다.

결론: 만성 *C pneumoniae*감염은 동맥경화증 진행에 중요한 역할을 함을 알 수 있었으며. 그 기전에 MMP 및 그 조절인자인 EMMPRIN/MT1-MMP 체계가 관여함을 알 수 있었다.

핵심되는 말: 만성 *Chlamydia pneumoniae* 감염, 동맥경화증, MMP, EMMPRIN

Table 1. Seropositive rate of Ig G and Ig A antibodies against *Chlamydia pneumoniae*.

Antibodies	Group I	Group II	Group III	p-value			
				Group I vs II	Group I vs III	Group II vs III	Group I+II vs III
Ig G	59.8%	67.2%	47.4%	NS	0.041	0.004	0.010
Ig A	64.6%	74.5%	57.7%	NS	NS	0.011	NS
Ig G&A	56.7%	61.3%	43.3%	NS	0.033	0.010	0.011

X^2 test; NS, not significant ($p > 0.05$).

Table 2. Seropositive rates of Ig G and Ig A antibodies in Group I and III, subgrouped by known risk factors of CAD.

risk factor	Seropositivity (%)		p-value	OR(95% CI)	Adjusted OR(95% CI)
	Group I	Group III			
Age(year) ≥ 55	62.5	53.3	NS		
< 55	38.7	38.8	NS		
Male	59.4	52.7	NS		
Female	52.1	31.0	0.035	2.4	5.2
Smoker	59.3	56.5	NS		
Nonsmoker	54.4	31.4	0.008	2.6	3.9
Hypertension	52.3	46.7	NS		
Normotensive	61.5	42.7	0.013	2.1	
Diabetes	47.8	70.0	NS		
Non-diabetes	59.9	40.2	0.004	2.2	
T-chol(mg/dl) ≥ 240	35.0	53.3	NS		
< 240	58.6	41.5	0.016	2.0	
HDL-chol(mg/dl) ≤ 35	57.6	55.6	NS		
> 35	58.1	42.0	0.038	1.9	
LDL-chol(mg/dl) ≥ 160	52.6	56.3	NS		
< 160	58.3	40.7	0.018	2.0	

X^2 test; logistic regression test; NS, not significant ($p > 0.05$); OR, odds ratio

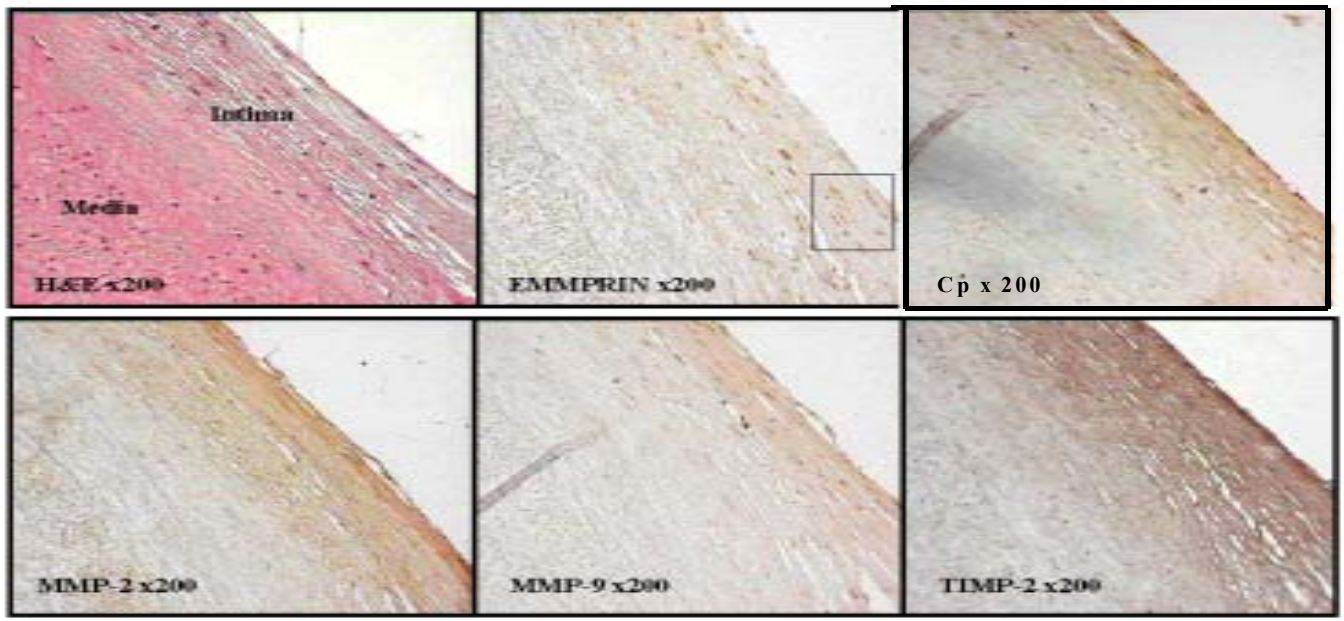

Fig. 1. Hematoxylin & Eosin stain, immunochemistry for MMP-2, MMP-9, TIMP-2, EMMPRIN and *Chlamydia pneumoniae* Ab. Sections of aorta taken from traumatic dissection with *C pneumoniae* seoronegativity shows no significant histological evidence of atherosclerosis. No immunoreactivity for anti-*C pneumoniae* Ab, MMP-2, MMP-9, TIMP-2 and trace immunoreactivity for EMMPRIN (rectangle) are shown. (Cp; *pneumoniae*)

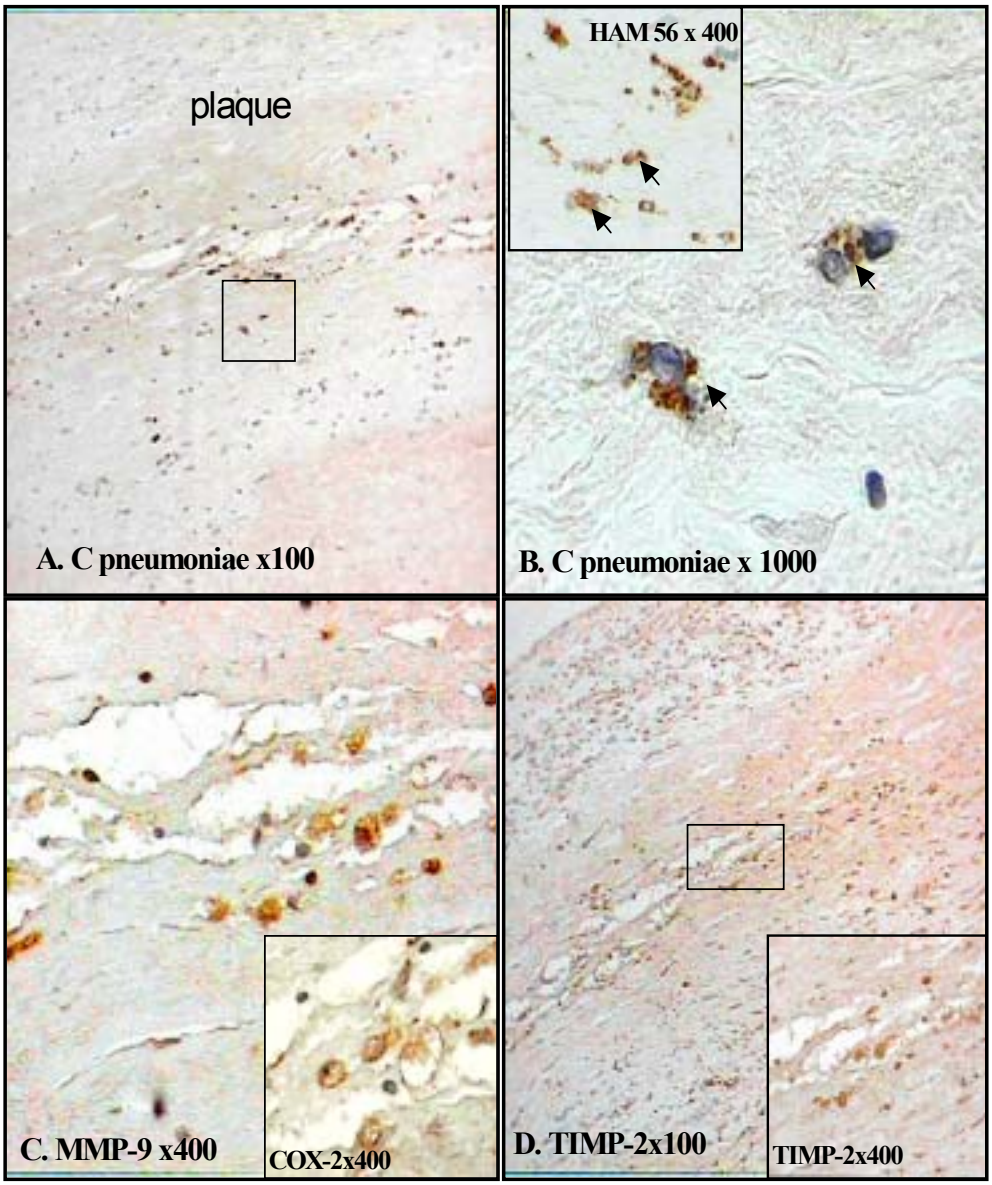

Fig. 2. Intracelluar *C pneueumoniae* are distributed in the base of atherosclerotic plaque (panel A, x100) and the immunoreactivity to *C pneumoniae* is primarily located in the macrophage/ mononuclear cells (panel B). The small box in panel B indicates the macrophage-rich region sampled from panel A and put into a high power view to define colocalization between intracellular *C pneumoniae* and tissue macrophage/mononuclear cells (small box in panel B, x 400, immunostaining with HAM56). The arrow in panel B indicates macrophages (HAM 56+) that are stained positively to *C pneumoniae.* Expression of MMP-9 (panel C), COX-2 (small box in panel C, x400), and TIMP-2 (panel D) show clocalization of immunoreactivity between *C pneumoniae*, COX-2, MMP-9 and its inhibitor (TIMP-2).

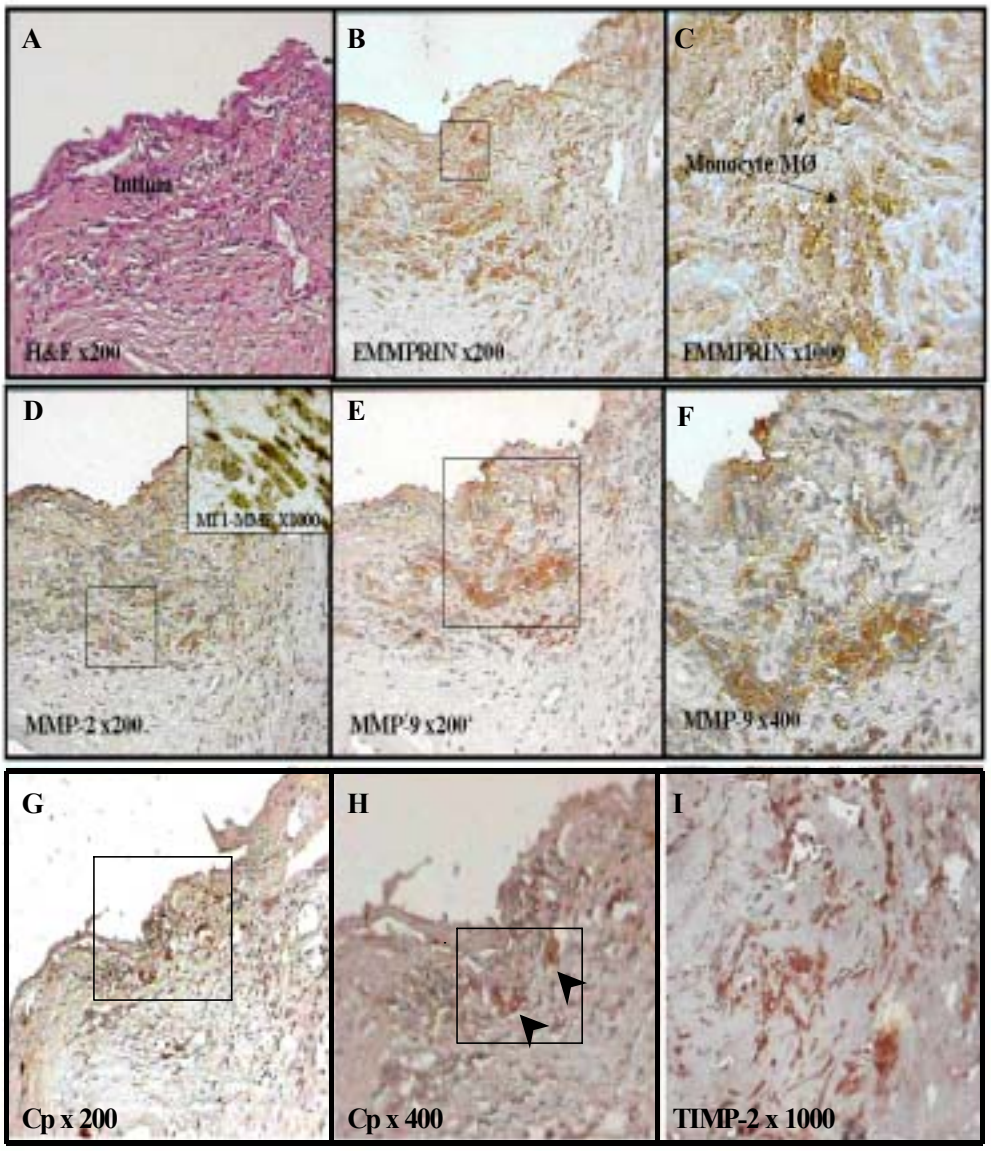

Fig. 3. Hematoxylin & Eosin stain of atheromatous plaque of carotid artery obtained from *C pneumoniae* seropositive patient shows prominent inflammatory infiltration with mononuclear cell and foam cells (panel A). In the same area with panel A, increased EMMPRIN, MMP-2, MMP-9 and MT1-MMP Immunoreactivities (dark brown color) are found in a similar pattern and distribution (panel B-F, Mϕ; macrophage) with the area stained by *C pneumoniae* (panel G & H, arrowhead, Cp ; *C pneumoniae*). Put into a high power view of small box in panel H shows immunoreacticity of TIMP-2 (panel I).

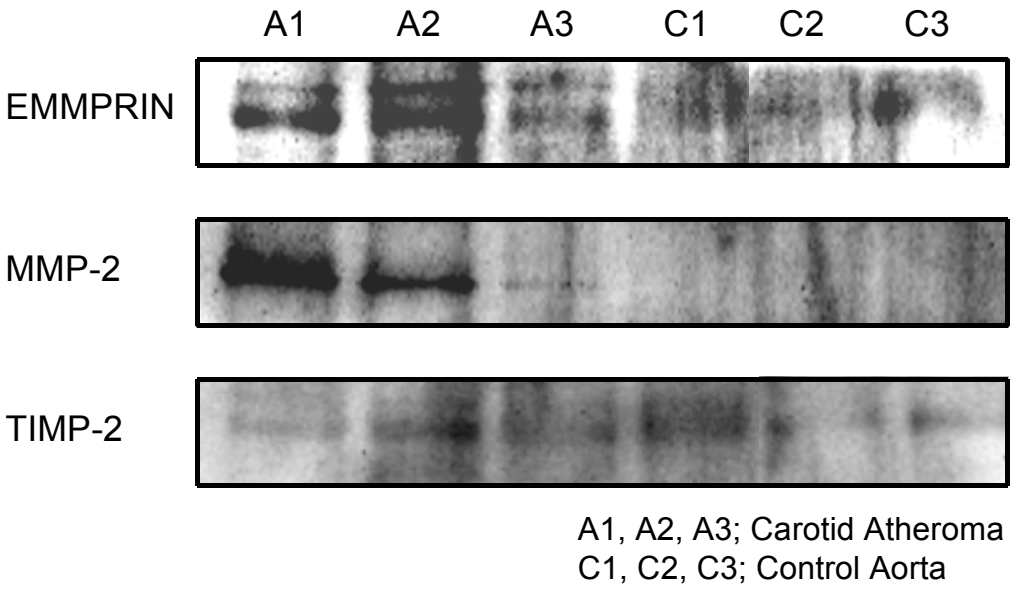

Fig. 4. Western blot for EMMPRIN, MMP-2, TIMP-2. EMMPRIN, MMP-2 and TIMP-2 are detected more prominent in *C pneumoniae* infected atheromatous tissues compared with control tissues.

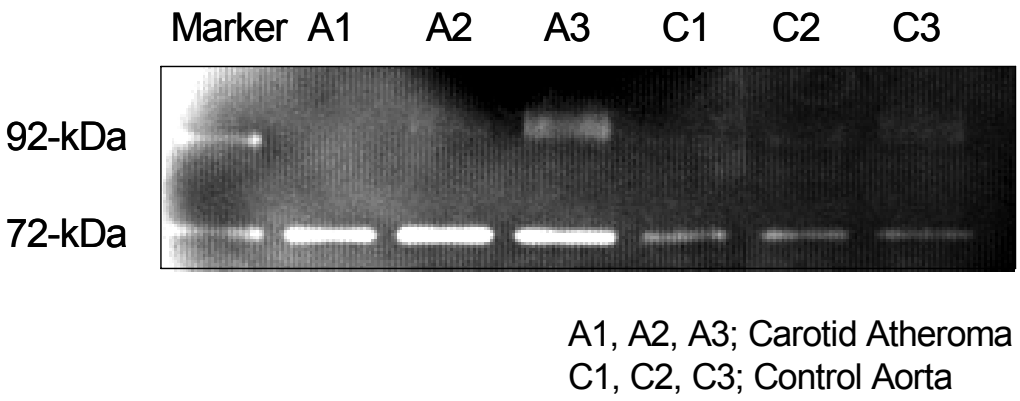

Fig. 5. Gelatine zymography for detection of MMP-9 and MMP-2 in infected atheromatous plaques. A 72-kDa band corresponding to MMP-2 and fainter 92-kDa band correponding to MMP-9 appeared in *C pneumoniae* infected atheromatous plaques. Gelatinolytic activity of MMP-2 is more prominent than that of MMP-9. But weak or no gelatinolytic activity is seen in control data.