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Upregulation of extracellular matrix metalloproteinase inducer (EMMPRIN) and gelatinases in human atherosclerosis infected with *Chlamydia pneumoniae*: The potential role of *Chlamydia pneumoniae* infection in the progression of atherosclerosis

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Abbreviations: CAD, coronary artery disease; COX-2, cyclooxygenase-2; EMMPRIN, extracellular matrix metalloproteinase inducer; HSP60, heat shok protein 60; IHC, immunohistochemistry; MMP, matrix metalloproteinase; MT1-MMP, membrane-type 1 matrix metalloproteinase; NF-κB, nuclear factor-κB; TIMP, tissue inhibitor of metalloproteinase; TNF, tumor necrosis factor

Abstract

***Chlamydia pneumoniae* infection implicated as an important etiologic factor of atherosclerosis, especially in coronary artery disease (CAD), was found *in vitro* to be associated with the induction of matrix metalloproteinases (MMPs). An extracellular matrix metalloproteinase inducer (EMMPRIN)/membrane-type 1 matrix metalloproteinase (MT1-MMP) system which induces and activates MMPs, is suggested to be functional and were upregulated in the failing myocardium. However, the upstream regulation of MMPs by *C. pneumoniae* within atheroma itself remains unclear. We evaluated the seroepidemiologic study of *C. pneumoniae* infection in CAD patients ($n = 391$) and controls ($n = 97$) and performed histopathological and *in vitro* analysis in atherosclerotic vascular tissues obtained from patients with seropositive to *C. pneumoniae* ($n = 20$), by using immunochemistry for *C. pneumoniae*, EMMPRIN/MT1-MMP, MMP-2, and MMP-9. The seropositive rates of both anti-*C. pneumoniae* IgG and IgA were 56.7% in CAD group and 43.3% in control group (P=0.033). Seropositive rate was increased in subgroups of CAD patients without conventional coronary risk factors compared to those with conventional risk factors. Immunoreactivities of EMMPRIN, MT1-MMP, MMP-2, and MMP-9 were increased in the atheromatous plaque itself, predominantly in immunoreactive macrophages/mononuclear cells to *C. pneumoniae*. Furthermore, Western blot analysis showed that EMMPRIN and MMP-2 were detected more prominently in atherosclerotic tissues infected with *C. pneumoniae* compared to control tissues. Zymographic analysis revealed that activities of MMP-2 and MMP-9 were more increased in atherosclerotic tissues infected with *C. pneumoniae* compared to control tissues. The present study demonstrated upstream regulation of MMPs can be induced by *C. pneumoniae* within atheromatous plaque itself. These findings help to understand the potential role of *C. pneumoniae* in the progression of atherosclerosis.**

Keywords: arteriosclerosis; chlamydia; enzyme induction; matrix metalloproteinases; tissue inhibitor of metalloproteinases

Introduction

Injury to a vessel wall and the associated inflammatory response are now generally known as essential components of atherogenesis. However, the stimuli that initiate and sustain the inflammatory process have not been fully identified. Infectious insult could be a candidate trigger of immuno-inflammatory response, and might be a source of chronic local or systemic inflammation (Braunwald *et al.*, 1997; Epstein *et al.*, 1999). Several infectious agents have been suggested as being responsible for chronic inflammation including *Cytomegalovirus, Helicobacter pylori* and *Chlamydia pneumoniae* (Ridker *et al.*,

1998). Among these agents, recently, substantial seroepidemiologic and experimental evidence demonstrated *C. pneumoniae* infection associated with the development and progression of atherosclerosis (Leinonen *et al.*, 1993; Song *et al.*, 2000). However, the mechanisms by which infectious agent affects the development and progression of atherosclerosis remain poorly understood. *C. pneumoniae* is an obligate, intracellular, Gram-negative bacterium, which commonly causes chronic persistent infection with metabolically quiescent and non-replicable. Chronic persistent infection of *C. pneumoniae* expresses basal levels of two major antigens: the major outer membrane protein (MOMP) and the heat shock protein 60 (HSP 60). Although *C. pneumoniae* can infect most cells present in atheroma (Beatty *et al.*, 1993; Campbell *et al.*, 1995; Godzin *et al.*, 1995; Gaydos *et al.*, 1996), it localizes mainly to macrophages/monocytes in atherosclerotic plaque. Macrophages/monocytes within atherosclerotic plaque produce matrix metalloproteinases (MMPs) (Galis *et al.*, 1995), enzymes now accorded a major role in the degradation of the extracellular matrix of vascular tissue (Libby *et al.*, 1995). Thus, macrophage/monocytes-derived MMPs may play a key role for plaque vulnerability, mineralization and subsequent thrombosis, and ultimately for the progression of atherosclerosis and the acute coronary syndrome (Davies *et al.*, 1985; Park *et al.*, 2001; Tintut *et al.*, 2002). Recently, chlamydial HSP 60 stimulated the expression of tumor necrosis factor-α (TNF-α) and MMP-9 by mouse peritoneal macrophages (Kol *et al.*, 1998; Pockley, 2002) and *C. pneumoniae* proteins induced the secretion of the 92-kDa gelatinase (MMP-9) in cell culture study with monocyte-derived macrophages (Kreula *et al.*, 2001). However, it remains unclear whether MMPs are regulated by macrophages/monocytes infected with *C. pneumoniae* and furthermore the upstream regulation of local MMP induction/EMMPRIN is associated with *C. pneumoniae* exist within atheromatous plaque itself.

Recently, a tumor-derived protein, extracellular matrix metalloproteinase inducer (EMMPRIN) and membrane bound MMP (MT1-MMP) were found to induce the production of MMPs from stromal fibroblasts, which would be crucial in tumor invasion (Biswas *et al.*, 1995; Chai *et al.*, 1997). EMMPRIN is a 58-kDa, membrane-bound protein that has been identified in both normal and diseased human tissue. It is also known as basign or CD147, glycoprotein, which is enriched on the surface of tumor cells, and which stimulates the production of several MMPs 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 various tissues, suggesting that this transmembrane protein has multiple roles (Li *et al.*, 2001). In addition, it has been reported that expression of EMMPRIN and MMP-9 were increased in the left ventricular myocardium of ischemic and nonischemic cardiomyopathy (Spinale *et al.*, 2000). Moreover, it has been reported that EMMPRIN is induced upon monocyte differentiation and is expressed in human atheroma (Major *et al.*, 2000).

Therefore, it would be important to investigate the molecular mechanism by which *C. pneumoniae* plays a major role in atherogenesis and to understand the mechanisms of the epidemiologic and pharmacologic links between this infectious agent and the clinical manifestations of atherosclerosis.

In the present study, we evaluated the seroepidemiologic relationship between *C. pneumoniae* and human atherosclerosis. To investigate the upstream regulation of MMPs induced by *C. pneumoniae* in atherosclerotic plaque itself, we performed histopathological and *in vitro* analyses in atherosclerotic vascular tissues, obtained from patients who were found seropositive for *C. pneumoniae*, by using antibodies to *C. pneumoniae*, EMMPRIN/MT1-MMP, MMP-2, and MMP-9.

Materials and Methods

Seroepidemiologic study

391 patients with typical symptoms of angina and with positive results in non-invasive testing (EKG, Treadmill test) who visited Yong-Dong Severance Hospital, who underwent coronary angiogram were included in this study. Among them, the patients who demonstrated more than 50% luminal narrowing 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 artery disease (CAD) and had normal findings on noninvasive tests for CAD, grouped into the negative control group (group III, n = 97). Serologic tests for anti-chlamydial IgG and IgA were performed using ELISA kit (Bioclonic, Sydney, Australia).

Tissue preparation and histologic examination

The study population consisted of 5 patients (range of age, 57-74 years; mean age, 67 years) with atherosclerotic aortic aneurysm dissection and dissection of aorta and 15 patients (range of age, 49-62 years; mean age, 56 years) with carotid artery diseases (n = 15), who were all referred to Yong-Dong Sever-

ance Hospital for evaluation and surgical treatment. All patients were seropositive to *C. pneumoniae* either with IgG or IgA antibodies. For control studies, aorta specimens, from which atherosclerotic lesions including fatty streak and plaque were excluded, were obtained from 5 patients (range of age, 18-29 years) who were surgically treated for traumatic aortic dissection with seronegative *C. pneumoniae*. Immediately after the careful removal of the specimen along with adjacent tissues and rinsing with phosphate buffered saline (PBS), each specimen was fixed with buffered 10% formalin to maintain morphologic integrity. Each segment was embeded in paraffin and cut in 5 μm sections, which were then stained with hematoxylin-eosin (H&E). Sections of these tissues were also used for the immunohistochemical staining procedure. One lesion from each section which had morphological characteristics of atherosclerosis ranging from fatty streak to complicated atherosclerotic lesion was assigned for histopathologic analysis and matched with the corresponding lesions for immunohistochemistry, respectively.

Immunohistochemical staining

Mouse anti-*C. pneumoniae* monoclonal antibody (RR-402) (DAKO, Carpenteria, CA) and goat polyclonal antibodies against human EMMPRIN, MT1-MMP, MMP-2, MMP-9, tissue inhibitor of metalloproteinase-1 (TIMP-1), TIMP-2, and COX-2 (cyclooxygenase-2) (Santa-Cruz Biotechnology, Santa Cruz, CA) were used as the primary antibodies for immunochemistry. 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. 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.

The paraffin sections were deparaffinized and rehydrated and then the sections are boiled with citric acid for 5 min in order to suppress nonspecific binding of the antibodies and to increase the exposure of antigens, and cooled at room temperature for 20 min. The sections were then treated with 0.3% H_2O_2 for 5 min to suppress endogenous peroxidase activity. After treatment with PBS (pH 7.2-7.4) for 5 min and application of 1:5 diluted anti-chlamydial primary antibodies (RR-402) and 1:100 diluted EMMPRIN, MT1-MMP, MMP-2, MMP-9, TIMP-1, TIMP-2, cyclooxygenase-2 (COX-2) and HAM56 primary anRibodies, the sections were incubated in a moist chamber for 1 h. After washing and bathing for 5 min 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 min 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) and DAB solution (Research Genetics, Huntsville, AL). The sections were then counterstained with Mayer's hematoxylin.

Western blot

A 50 μg proteins were subjected to 11% gradient SDS-PAGE gel and transferred to immunobilon-P membrane (Millipore, Bedford, MA) at 12 V for 1 h. The membrane was blocked in 5% non-fat dry milk in TBST at 25°C for 1 h. Proteins were detected using EMMPRIN, MMP-2 and TIMP-2: 5 μg/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).

Gelatine zymography

Enzymatic activities of MMP-2 and MMP-9 were investigated using zymographic analysis. The protein content of the atherosclerotic and control tissues were calculated by Bradford method, using bovine serum albumin as a standard. 50 μg of proteins from atherosclerotic aortic and carotid tissues were loaded on an 11% SDS-PAGE gel containing with 0.1% gelatin for electrophoresis under 4°C cold room. Gels were reacted with collagenase buffer for 16 h at 37°C, stained with 0.25% Coomassie brilliant blue, and destained with 30% isopropanol in 10% acetic acid to visualize the MMP bands.

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.

Results

Seroepidemiologic study

A total of 488 persons were included in this seroepidemiologic study for anti-*C. pneumoniae* IgG and IgA (group I: 254 in the disease, group II: 137 in the positive control, group III: 97 in the negative control). Simultaneous seropositive rates of both IgG and IgA were 56.7%, 61.3%, and 43.3% in group I, II and III,

Table 1. Seropositive rate of IgG and IgA antibodies against *Chlamydia pneumoniae*

Antibodies	Group I	Group II	Group III	*P*			
				I *vs.* II	I *vs.* III	II *vs.* III	I+II *vs.* III
IgG	59.8%	67.2%	47.4%	NS	0.041	0.004	0.010
IgA	64.6%	74.5%	57.7%	NS	NS	0.011	NS
IgG, IgA	56.7%	61.3%	43.3%	NS	0.033	0.010	0.011

X^2 test; NS, not significant ($P>0.05$). Group I, patients who demonstrated more than 50% luminal narrowing in at least one vessel ($n=254$); gropu II, patients who had symptom of angina but normal coronary arteries or minimal lesion ($n=137$); group III, patients who had not experienced any symptoms related to CAD and had normal findings on noninvasive tests for CAD ($n=97$).

Table 2. Seropositive rates of IgG and IgA antibodies in Group I and III, subgrouped by known risk factors of CAD

Risk factor	Seropositivity (%)		*P*	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.

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 according to 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=2.0) 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).

Immunohistochemical staining

Sections of aorta taken from traumatic dissection as the normal control showed no histological evidence of atherosclerosis except for minimal intimal thickening, and normal patterns of elastic media. There was no immunoreactivity to *C. pneumoniae* and trace immunoreactivities for MMP-2, MMP-9, TIMP-2 and

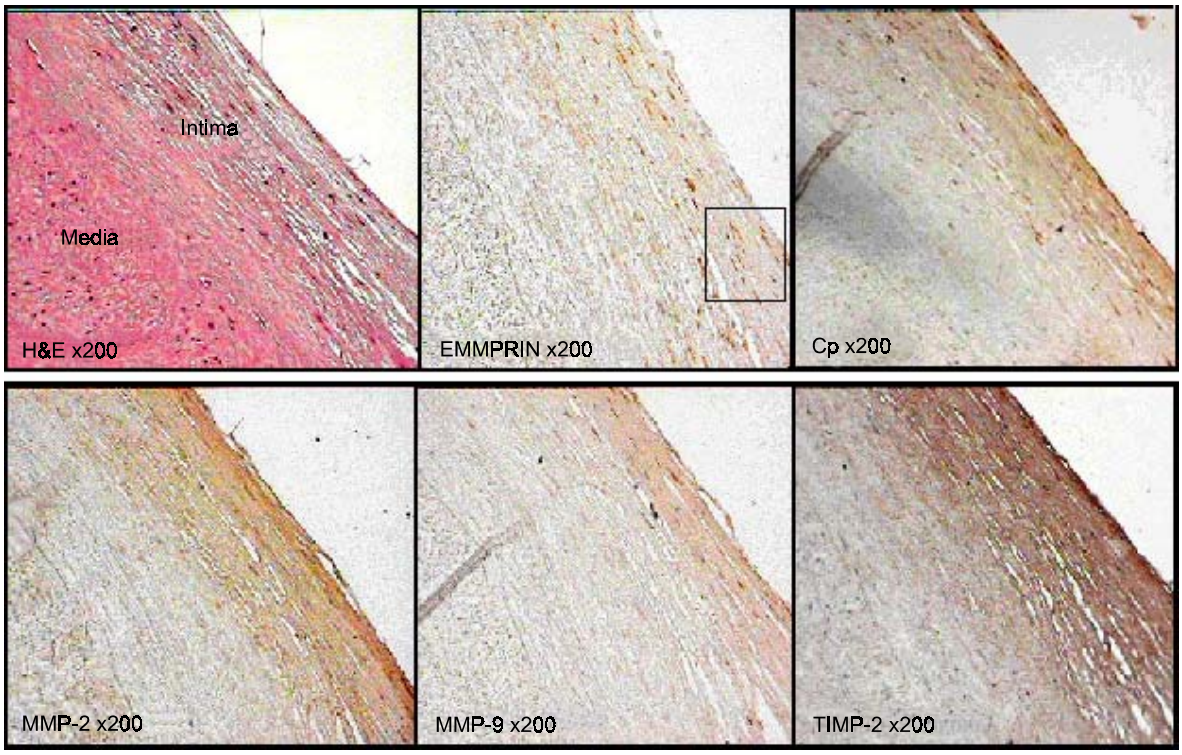

Figure 1. Hematoxylin and eosin (H&E) stain and immunohistochemical staining for EMMPRIN, *C. pneumoniae*, MMP-2, MMP-9 and TIMP-2. Sections of aorta taken from traumatic dissection with seronegative *C. pneumoniae* shows no significant histological evidence of atherosclerosis except for minimal intimal thickening. No immunoreactivity for anti-*C. pneumoniae* Ab and trace immunoreactivity for EMMPRIN (rectangle), MMP-2, MMP-9 and TIMP-2 are shown. Cp, *C. pneumoniae*

EMMPRIN in the minimal thickened intima (Figure 1). In contrast to the control group, the 20 case-specimens showed a thickened intima from necrosis and a lipid-laden plaque formation that characteristic atherosclerotic aortas and carotid arteries. There was also a prominent inflammatory infiltration of mononuclear and foam cells in the atheromatous plaques. *C. pneumoniae* was stained dark brown within atheromatous plaques in 12 of 20 atheromatous tissues, mainly in tissue macrophages/mononuclear cells. Intracelluar *C. pneumoniae* were distributed in the base of atherosclerotic plaque (Figure 2, panel A) and the immunoreactivity to *C. pneumoniae* was primarily colocalized in tissue macrophage/mononuclear cells (Figure 2, panel B). Expression of MMP-9, COX-2 and TIMP-1 showed colocalization of immunoreactivity between *C. pneumoniae* (Figure 2). In the atherosclerotic lesions, immunoreactivity for EMMPRIN, MT1-MMP, MMP-2, and MMP-9 were evident in all cases along with plaques, primarily in tissue macrophages/mononuclear cells, intimal and medial smooth muscle cells. Furthermore, increased EMMPRIN, MMP-2, and MMP-9 immunoreactivities were found to have a similar pattern and colocalization within atheromatous plaque stained by *C. pneumoniae* (Figure 3).

Western blot analysis

Western blot analysis was performed to define the expression of these proteins quantitatively and exclude cross reactivity by immunohistochemical staining. EMMPRIN, MMP-2 and TIMP-2 (data are not shown) were more prominently detected in *C. pneumoniae* infected atheromatous specimens ($n = 20$) than in control specimens ($n = 5$) (Figure 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 significant comparison with those of control specimen quantitatively (Figure 5). Futhermore, gelatinolytic activity of MMP-2 was more prominent than that of MMP-9 in *C. pneumoniae* infected atheromatous tissue which was similar to EMMPRIN, MMP-2/TIMP-2 in western blot analysis, suggestive of the upstream cellular and molecular mechanisms for a local MMP induction/activation system.

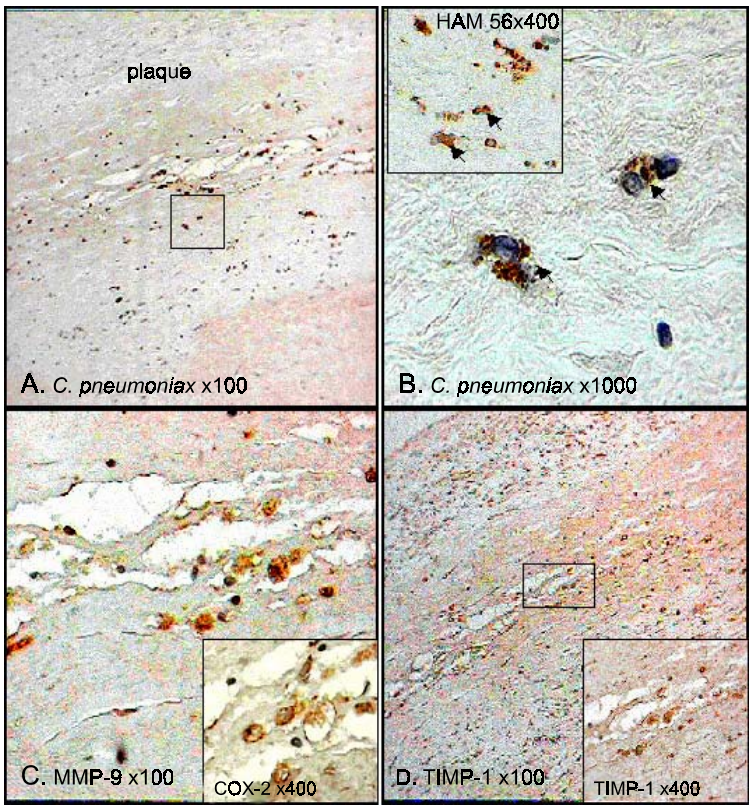

Figure 2. Intracelluar *C. pneumoniae* are distributed in the base of atherosclerotic plaque (panel A, ×100) and the immunoreactivity to *C pneumoniae* is primarily located in the macrophage/mononuclear cells (panel B). The small box in panel B indicates the macrophage-rich region sampled from panel 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, ×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, ×400), and TIMP-1 (panel D) show clocalization of immunoreactivity between *C. pneumoniae*, COX-2, MMP-9 and its inhibitor (TIMP-1).

Discussion

In the present study, we evaluated the seroepidemiologic relationship between *C. pneumoniae* and human atherosclerosis. The seropositive rates of both anti-*C. pneumoniae* IgG and IgA were higher in CAD group than in control group. Seropositive rate was increased in subgroups of CAD patients without conventional coronary risk factors compared to those with conventional risk factors. Therefore, *C. pneumoniae* may play a pathobiologic role in atherosclerosis in those with low risk factors. To define the pathobiologic role in atherosclerosis and investigate the upstream regulation of MMPs induced by *C. pneumoniae* in atherosclerotic plaque itself, we performed histopathological and *in vitro* analysis in atherosclerotic vascular tissues obtained from patients with seropositive to *C. pneumoniae*, by using immunochemistry for *C. pneusmoniae*, EMMPRIN/MT1-MMP, MMP-2, and MMP-9. Immunoreactivities of EMMPRIN, MT1-MMP, MMP-2, and MMP-9 were increased in the atheromatous plaque itself, predominantly in immunoreactive macrophages/mononuclear cells to *C. pneumoniae*. Western blot analysis showed that EMMPRIN and MMP-2 proteins were more prominent in atherosclerotic tissues infected with *C. pneumoniae* compared to control tissues. Zymographic analysis revealed that activities of MMP-2 and MMP-9 were more increased in atherosclerotic tissues infected with *C. pneumoniae* compared to control tissues. Furthermore, gelatinolytic activity of MMP-2 was more prominent than that of MMP-9 in *C. pneumoniae* associated atherosclertotic tissue. Because EMMPRIN mainly induces MMP-2 rather than MMP-9, this result indirectly suggests that EMMPRIN palys a more important role in the MMP mediated atheroma remodeling in *C. pneumoniae* infection. The exact mechanism how *C. pneumoniae* triggers the MMP regulation system is uncertain. But, possible mechanism is that chlamydial antigen, such as HSP 60 or outer membrane protein, binds immunoglobulin domain of EMMPRIN and thereby activates EMMPRIN. The present study demonstrated the up-

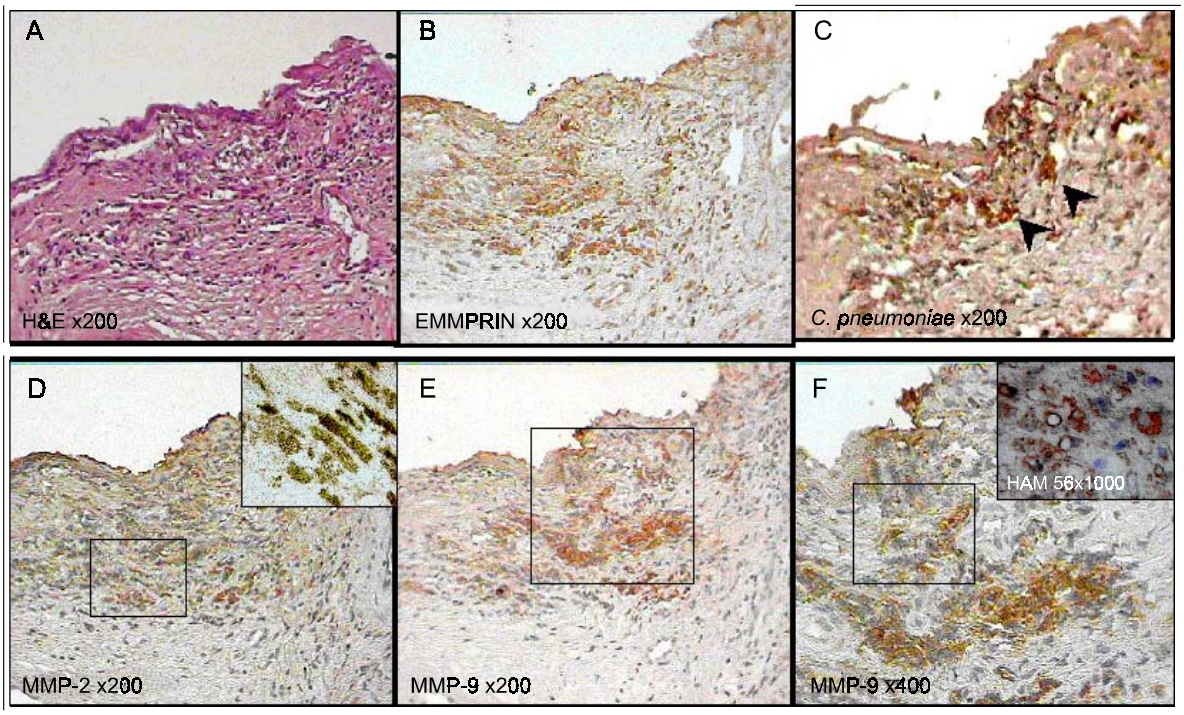

Figure 3. Hematoxylin and eosin (H&E) stain of atheromatous plaque of carotid artery obtained from *C. pneumoniae* seropositive patient shows prominent inflammatory infiltration with mononuclear cell and foam cells (panel A). In the same area with panel A, increased EMMPRIN (panel B), MMP-2 (panel D), MMP-9 (panel E) and MT1-MMP (box of panel D, ×1000) immunoreactivities (dark brown color) are found in a similar pattern and distribution with the area stained by *C. pneumoniae* (panel C, arrowhead). A high power view of small box in panel E shows immunoreactivity of MMP-9 (panel F). The small box in panel F indicates tissue macrophage/mononuclear cells to define colocalization with intracellular *C. pneumoniae* in panel C (small box in panel F, ×1000, immunostaining with HAM 56).

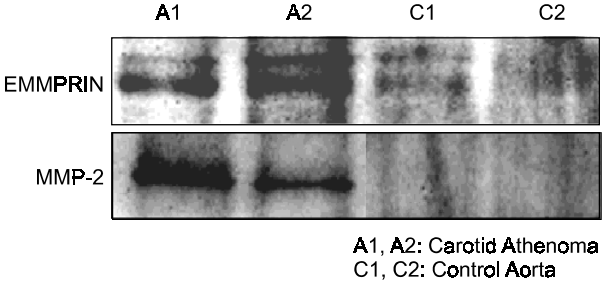

Figure 4. Western blot for EMMPRIN and MMP-2. EMMPRIN and MMP-2 are detected more prominent in *C. pneumoniae* infected atheromatous tissues compared with control tissues.

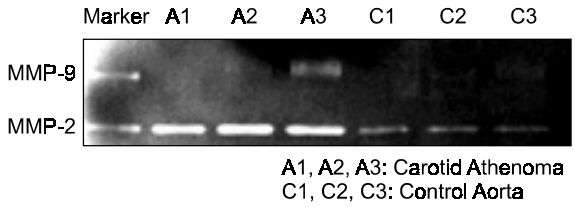

Figure 5. Gelatin zymography for detection of MMP-9 and MMP-2 in infected atheromatous plaques. A 72-kDa band corresponding to MMP-2 and fainter 92-kDa band corresponding to MMP-9 appeared in *C. pneumoniae* infected atheromatous plaques ($n = 20$). Gelatinolytic activity of MMP-2 is more prominent than that of MMP-9. But weak or no gelatinolytic activity is seen in control aorta ($n = 5$).

stream regulation of MMPs induced by *C. pneumoniae* within atheromatous plaque itself. These findings help to understand the potential role of *C. pneumoniae* in the progression of atherosclerosis

Although it is tempting to consider *C. pneumoniae* infection as a possible primary cause of atherosclerotic lesion formation in some cases (Rupprecht *et al.*, 2001), the currently available data do not justify this conclusion. Area of *C. pneumoniae* infection in vessel wall is generally focalized and does not affect all lesions examined, raising some questions about the specificity and the biological significance of involved sites. However, a recent autopsy data showed increased frequency of chlamydial antigens in the cardiovascular tissues of patients who had died of ischemic heart disease than other disease (64% versus 38%). Moreover, the effects of a focal infection might influence the pathobiology of the surrounding atherosclerotic environment (Jackson *et al.*, 1997). Many other seroepidemiologic and histopathologic

results support an association between *C. pneumoniae* infection and atherosclerosis, but the pathogenic mechanism is still unclear.

Chlamydial HSP 60 induces the secretion of 92-kDa gelatinase (MMP-9) and the production of TNF-α in culture study with human monocyte-derived macrophages (Kol *et al.*, 1998), and *C. pneumoniae*, when present in a macrophage-containing inflammatory environment, actively participate in the destruction of the extracellular matrix (Kreula *et al.*, 2001). In the present study, most of the cases showed increased the expression of inflammatory mediators, such as EMMPRIN, MMPs and COX-2, with colocalized with the immunoreactivity of *C. pneumoniae*. 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, suggesting potential role of these enzymes for atherosclerotic disease.

COX-2 is another enzyme regulated by NF-κB, is responsible for the increased production of prostaglandins and thromboxane in inflammatory disease. The induction of COX-2 in monocyte and subsequent production of prostaglandin E_2 have triggered MMPs signal transduction pathway. A possible pathophysiologic mechanism supporting these results is that the promoter region of gelatinase gene contains NF-κB 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 probably through the activation of NF-κB. This possibility is supported by a recent observation that *C. pneumoniae* infection activates NF-κB in vascular smooth muscle cells and endothelial cells (Dechend *et al.*, 1999). The factors regulating the production of these MMPs in atherosclerotic lesions are poorly understood. However, it has been known that the activity of MMPs on substrates of the extracellular matrix depends on a balance between these enzymes and their endogenous inhibitors, TIMPs. Since TIMP expression can be regulated by 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 1:1 molar ratio required for full inhibition of gelatinase. Although the possibility of an innocent bystander effect, these findings suggest that *C. pneumoniae* might increase the capacity of tissue macrophages/mononuclear cells to produce gelatinase by inducing the upstream regulation of local gelatinases/EMMPRIN within atheromatous plaque itself. Increased expression of gelatinase might result in subsequent increment of proteolytic activity in the vascular microenvironmental melliu and lead to enhance the phenotypic change and migration of vascular smooth muscle cells and extracellular remodeling in the pathogenetic process of atherosclerosis (Kreula *et al.*, 2001). However, these results may contain some limitations, for example, the present study did not include in vitro studies with a purely infected monocytes/macrophage cell line, but rather to use human atheromatous plaques which are heterogenous cell line, and therefore many other factors, such as other infectious burden (cytomegalovirus and herpes simplex virus ,etc), could have affected our results. But, in this study, all *C. pneumoniae* antigens were adjacent to macrophage/monocyte, MMP-2 and MMP-9 and EMMPRIN in the atherosclerotic tissues and *C. pneumoniae* antibody (IgG and IgA) seropositivity was closely correlated with CAD. So, the possibility that other infectious burdens affect the result is low.

Despite these limitations, our results suggested that not only MMP-2 (72-kDa gelatinase) but also MMP-9 (92-kDa gelatinase) might play an important role in the interaction of cell-extracellular matrix and the extracellular remodeling of atheromatous tissues infected with *C. pneumoniae*.

However, it remains unclear whether the upstream cellular and molecular mechanism of local MMP induction/activation system actually exists. Recently, Spinale *et al.* demonstrated that MMP induction/activation system (EMMPRIN and MT1-MMP) exists in the human LV myocardium, which is upregulated in failing myocardium (Spinale *et al.*, 2000). Moreover, EMMPRIN is induced on differentiating monocytes and is expressed in human atheroma (Major *et al.*, 2000). They suggested: 1) monocyte to macrophage differentiation induces both EMMPRIN and MMP expression, 2) EMMPRIN may play a role in atherosclerotic lesion formation and the influx/differentiation of monocyte may be responsible for destabilization of atheroma. Berditchevski and colleagues demonstrated that EMMPRIN forms a complex with α3β1 integrin (Berditchevski *et al.*, 1997), which functions for cell-cell, cell-extracellular matrix adhesion and the transduction of cellular signaling cascades. The coexistence of EMMPRIN and α3β1 integrin suggests that EMMPRIN mediated MMP induction may be influenced by 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 protein kinase C (PKC) phosphorylation site, which may also be an important intracellular regulatory mechanism (Lam *et al.*, 1998). *In vitro* studies have demonstrated that although EMMPRIN induces MMP expression, it does not in-

fluence the basal expression of TIMP (Guo *et al.*, 1997).

In the present study, MMPs were regulated by macrophages/monocytes infected with *C. pneumoniae* and the upstream regulation of local gelatinases induction/EMMPRIN was associated with *C. pneumoniae* existed within atheromatous plaque itself. Thus, increased expression of EMMPRIN in tissue macrophages/mononuclear cells of infected atherosclerotic plaque might contribute to increased expression of MMPs, 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. These finding suggests that *C. pneumoniae* may play an important role in atherosclerosis. Possible mechanisms involve infected macrophage differentiation and the induction of EMMPRIN/gelatinases may 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 about EMMPRIN-mediated MMP upstream regulation are needed to enlighten the potential role of EMMPRIN in the development and progression of atherosclerosis.

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