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# The Potential Roles of Cyclooxygenase-2 and Matrix Metalloproteinase-9 in Cytomegalovirus-Infected Atherosclerotic Aorta and Coronary Artery

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Inflammation appears to have a major role in the development of atherosclerosis. Cyclooxygenase-2 (COX-2) is involved in the inflammatory response via the generation of prostanoids that, in turn, are involved in the production of matrix metalloproteinases (MMPs). This study hypothesized that a vascular infection with cytomegalovirus (CMV) may induce a chronic inflammatory reaction and activated inflammatory cells may express inflammatory mediators such as cyclooxygenase-2 (COX-2) and matrix metalloproteinases-9 (MMP-9). To confirm the hypothesis, the immunohistochemical stains for CMV late antigen, COX-2, MMP-9, macrophage, and T-lymphocyte were performed on CMV-infected atherosclerotic lesions. The immunoreactivity for COX-2 and MMP-9 was evident in all cases of atherosclerosis along with plaques, mainly in macrophages/foamy cells, intimal and medial smooth muscle cells, and endothelial cells of the intima. Within the intima, the increased immunoreactivity for COX-2 and MMP-9 was colocalized to the area stained with CMV late antigen. Sections from control specimens showed no immunoreactivity for CMV late antigen, COX-2 and MMP-9. These data seem to support the hypothesis that CMV may participate in a pathogenetic mechanism for atherogenesis or progression of atherosclerosis.

**Key Words:** Cytomegalovirus (CMV), Atherosclerosis, Cyclooxygenase-2 (COX-2), Matrix metalloproteinase-9 (MMP-9), Immunohistochemical stain

## INTRODUCTION

Conventional risk factors including hyperlipidemia, hypertension, diabetes, cigarette smoking, sex, and family history of premature vascular disease account only for approximately half of the patients with clinically apparent atherosclerosis[33]. As many as 50% of patients with atherosclerosis lack currently identified risk factors, an observation indicating that additional factors predisposing to atherosclerosis are as yet undetected. Recently, a potential link between infectious agents and atherosclerosis has been suggested. Data obtained from several seroepidemiological studies has given rise to the hypothesis that an infection can initiate or maintain the atherosclerotic process[10]. Several agents have been suggested as being responsible for chronic inflammation including cytomegalovirus, *Helicobacter pylori*, and *Chlamydia pneumoniae*[24,30,39].

The production of matrix metalloproteinases (MMPs) by human monocytes has been shown to occur through a prostaglandin (PG) $E_2$-cAMPs dependent pathway[31,44,45]. Signaling through this pathway involves the modulation of prostaglandin H synthase, that is, cyclooxygenase (COX)[9]. Two types of COX isoform have been identified, referred to as COX-1 and COX-2. In contrast to COX-1, which is a constitutively expressed enzyme involved in maintaining low levels of PG, COX-2 is induced in response to cell activations such as growth factors, cytokines, and phorbol esters, suggesting that this enzyme is involved in the generation of PG in inflammation. The induction of COX-2 in monocytes and the resulting production of $PGE_2$ have been shown to be involved in the signal transduction pathway leading to the production of MMPs by these cells[32].

In view of the interactions of COX-2 and MMPs, this study hypothesized that the inflammation via COX-2 and the production of MMPs might be involved in the development of atherosclerosis in human aorta and coronary artery.

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The aim of this study was to determine the cellular location of COX-2, and to investigate for codistribution of COX-2 and MMP-9. This study performed immunohistochemical staining on the atherosclerotic tissue sections obtained from paraffin embedded blocks that were positive to cytomegalovirus by *in situ* hybridization and polymerase chain reaction[12], by using antibodies to CMV late antigen, COX-2, MMP-9, macrophage and T-lymphocyte.

The purpose of this study was to determine the possible pathogenetic role of COX-2 and MMP-9 in cytomegalovirus-infected atherosclerotic aorta and coronary artery by histopathologic observation.

## MATERIALS AND METHODS

### 1. Materials

The study population consisted of 23 persons (15 men and 8 women; range of age, 21~65 years; mean age, 43 years) with atherosclerotic aorta and coronary artery, who were all referred to National Institute of Scientific Investigation (NISI; Seoul, Korea) for autopsy between April and October 2001. For control studies, aorta and coronary artery specimens, from which atherosclerotic lesions including fatty streak and plaque were excluded, were obtained from 10 persons (6 men and 4 women; range of ages, 18~54 years; mean age, 36 years) who were died from traffic accidents.

### 2. Tissue preparation

Immediately following removal of aortic and coronary artery segments, each segment was fixed with 10% formalin in order to maintain vascular morphological integrity. To preserve the integrity of the adventitia and perivascular tissues, aorta and coronary artery specimens were carefully removed in a segment along with adjacent tissues and rinsed with PBS (phosphate buffered saline). Each segment was overnight processed through the following solutions: serially 70%, 75%, 80%, 90%, 95% ethyl alcohol, absolute ethyl alcohol, acetone, 2 changes of xylene, 2 changes of paraffin by Hypercenter XP Tissue Processing System (Shandon® Scientific Limited, Cheshire, England). The processed segment was embedded in paraffin and cut in 5 μm sections, which were stained with hematoxylin-eosin (H&E). Sections of these tissues were also used for immunohistochemical staining. One lesion from each section, which had morphological characteristics of atherosclerosis ranging from fatty streak to complicated atherosclerosis lesion, was assigned for histopathologic analysis.

### 3. Immunohistochemical stain for CMV late antigen, COX-2, MMP-9, Macrophage, T-lymphocyte

Mouse anti-cytomegalovirus (CMV) late antigen monoclonal antibody (Chemicon International, Temecula, CA, USA), mouse anti-human cyclooxygenase-2 (COX-2) monoclonal antibody (BD Biosciences, San Jose, CA, USA), and rabbit anti-human matrix metalloproteinase-9 (MMP-9) polyclonal antibody (Chemicon International, Temecula, CA, USA) were used as the primary antibodies for immunohistochemical staining. To characterize the type of inflammatory cells, rabbit anti-human macrophage (CD68) monoclonal antibody (Lipshaw Immunon, Pittsburgh, PA, USA) and rabbit anti-human T-lymphocyte (CD45RO) monoclonal antibody (Lipshaw Immunon, Pittsburgh, PA, USA) were used for the immunohistochemical staining. Peroxidase-conjugated secondary antibodies were used with these primary antibodies.

The paraffin sections (5 μm) were made and transferred to poly-L-lysine coated glass slides (Polysine™, Portsmouth, NH, USA). The paraffin sections were deparaffinized and rehydrated through the following solution: xylene, 3 times for 3 minutes in each, and serially 100%, 95%, 80%, 75%, and 70% ethyl alcohol for 3 minutes in each. The sections were then treated with 3% $H_2O_2$ for 10 minutes to suppress endogenous peroxidase activity. The sections were boiled in plastic container which was filled with 10 mM sodium citrate, pH 6.0 for 5 minutes with microwave oven (800 W) to retrieve antigen sites, and cooled at room temperature for 20 minutes, followed by washing in TBS (Tris buffered saline; pH 7.2) for 5 minutes. Nonspecific binding was blocked by incubation with 10% normal goat serum for polyclonal antibodies and 10% normal horse serum for monoclonal antibodies. The sections were incubated at room temperature in moist chamber for 1 hour with primary antibodies (1:40 diluted anti-CMV late antigen, 1:50 diluted anti-MMP-9, 1:100 diluted anti-COX-2, 1:200 diluted anti-macrophage, 1:200 diluted anti-T-lymphocyte). After washing and bathing in TBS (Tris buffered saline; pH 7.2) for 5 minutes, the biotinylated secondary antisera cocktail including goat anti-mouse and anti-rabbit IgG diluted 1:400 was incubated on the slides for 20 minutes at room temperature

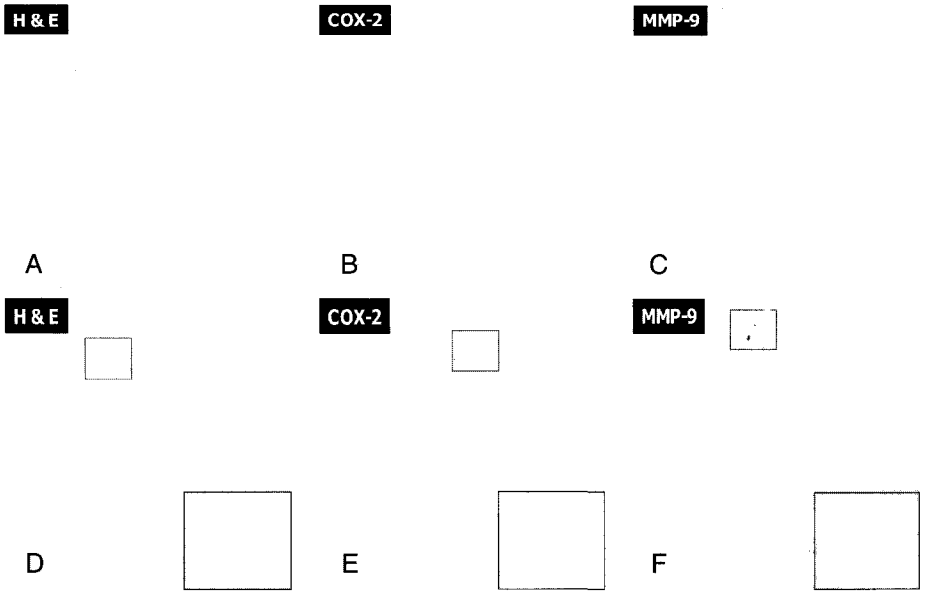


**Fig. 1.** Hematoxylin-eosin stain, immunohistochemical stain for COX-2 and MMP-9 of control (**panel A-C**, respectively, ×100) and atherosclerotic aortas (**panel D-F**, respectively, ×40; small box in panel, ×400). Normal aorta (**panel A**) showed normal pattern of elastic media. Compared with controls, there was prominent inflammatory infiltration with mononuclear cells and foam cells in the atherosclerotic plaque (**panel D**). In nonatherosclerotic section (**panel B & C**), immunoreactivity for COX-2 and MMP-9 were not shown. Atherosclerotic lesion (**panel E & F**) demonstrated strong immunoreactivity for COX-2 and MMP-9.

in a moist chamber. The sections were then processed by the streptoavidin-biotin-peroxidase complex method using the LSAB plus kit (DAKO Inc, Carpinteria, CA, USA), and stable AEC substrate solution (3-amino-9-ethylcarbazole) was used as a chromogen. Gill's hematoxylin was used as a counterstain, and sections were dehydrated, cleared, and mounted.

## RESULTS

### 1. Histopathologic analysis

The sections of aorta and coronary artery taken from control groups showed no histological evidence of atherosclerosis, except minimal intimal thickening, and normal patterns of elastic media (Fig. 1, panel A; Fig. 2, panel A). In contrast to the control group, lesions that ranged from fatty streak to complicated atherosclerotic plaque were noted in all persons with atherosclerosis, and showed a thickened intima and fragmented elastic lamina. There was also prominent inflammatory infiltration with mononuclear cells and foam cells in atherosclerotic plaque, especially in the central core, and to a lesser extent in the inner media and adventitia.

### 2. Immunohistochemical stain

Serial sections of each tissue samples (aorta and coronary artery) were examined sequentially for viral antigen, and for correlation of virologic results with histopathologic findings. Positive staining reactions observed in the aorta and coronary artery tissue sections with immunohistochemical stain are illustrated in Figs. 1 through 3. CMV was demonstrated in occasional cells in the luminal surface and in focal clusters of spindle-shaped or 'foamy' cells in the subendothelium as well as deeper intimal layers. Although the histogenesis of these cells has not been definitely established, they corresponded to endothelial cells, smooth muscle cells, or monocytes/macrophages on the basis of morphology and tissue location.

Sections from control specimens showed little immunoreactivity for CMV late antigen, COX-2, MMP-9, macrophage, and T-lymphocyte in the minimal thickened intima, and no immunoreactivity in the media (Fig. 1, panel B & C; Fig. 2, panel B & C). In atherosclerotic sections (plaque with central core), however, CMV late antigen was stained within the plaque macrophages/mononuclear cells (Fig. 3, panel A & D). The immunoreactivity for COX-2 and MMP-9 was evident in all cases of atherosclerosis along with

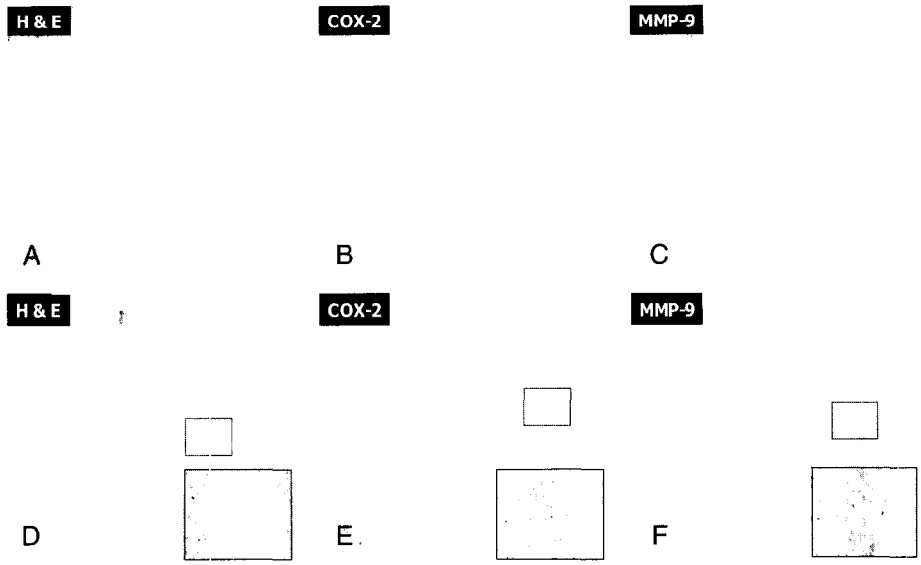


**Fig. 2.** Hematoxylin-eosin stain, immunohistochemical stain for COX-2 and MMP-9 of control (**panel A-C,** respectively, ×100) and atherosclerotic coronary artery (**panel D-F,** respectively, ×40; small box in panel, ×400). Normal coronary artery (**panel A**) showed normal pattern of elastic media. Compared with controls, there was prominent inflammatory infiltration with mononuclear cells and foam cells in the atherosclerotic plaque (**panel D**). In nonatherosclerotic section (**panel B & C**), immunoreactivity for COX-2 and MMP-9 were not shown. Atherosclerotic lesion (**panel E & F**) demonstrated strong immunoreactivity for COX-2 and MMP-9.

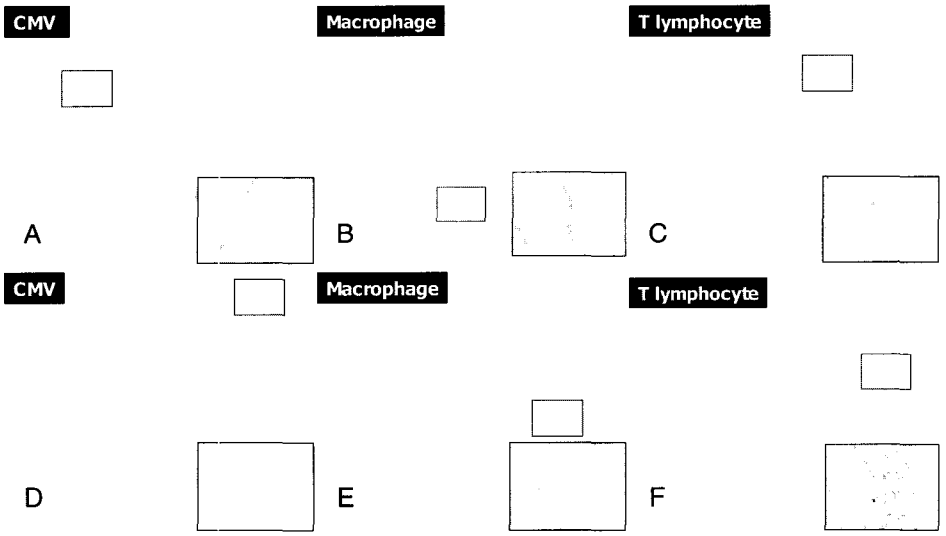


**Fig. 3.** Immunohistochemical stain for CMV late antigen, macrophage, T-lymphocyte of atherosclerotic aorta (**panel A-C,** respectively, ×40; small box in panel ×400) and coronary artery (**panel D-F,** respectively, ×40; small box in panel ×400). The immunoreactivity to CMV was primarily located in endothelial lining of the vascular wall (panel **A & D**). The immunoreactivity for macrophages (**panel B & E**) and for T-lymphocytes (**panel C & F**) was shown. Note the similar distribution of macrophages and T-lymphocytes in the coronary artery (**panel E & F**).

plaques, mainly in macrophages/foamy cells, intimal and medial smooth muscle cells, and endothelial cells of the intima (Fig. 1, panel E & F; Fig. 2, panel E & F). Within the intima, the increased immunoreactivity for COX-2, MMP-9 was colocalized to the area stained with CMV late antigen. Immunoreactive patterns of COX-2 and MMP-9 were similar. Immunoreactivity for macrophage and T-lymphocyte were found in a similar pattern and distribution with COX-2, MMP-9 (Fig. 3, panel B, C, E, and F). In fatty streak, immunoreactive patterns for COX-2, MMP-9, ma-

crophage, and T-lymphocyte were similar, although immunoreactivity was less prominent.

The immunoreactivity for COX-2 and MMP-9 increased in the atherosclerotic plaques itself, predominantly in the surrounding area of immunoreactive cytomegalovirus. These data seem to support that cytomegalovirus may participate in a pathogenetic mechanism for atherogenesis or progression of atherosclerosis.

## DISCUSSION

Atherosclerosis and its related diseases, in particular myocardial infarction (MI) and coronary heart disease (CHD), are a major cause of the morbidity and mortality worldwide. The differences in the prevalence of conventional atherosclerotic risk factors (such as smoking, hypertension, diabetes mellitus, and hypercholesterolemia) do not fully account for the variation in the prevalence or severity of atherosclerosis. Consequently, there is intense research interest focused on seeking other atherogenic risk factors. Current data supports the hypothesis that atherosclerosis is an inflammatory disease rather than a degenerative disease owing to hemodynamic loading[2,40] and studies examining markers of inflammation (e.g., C-reactive protein) demonstrate a relationship between an increasing inflammation and the risk of a vascular event. It is also recognized that plaque activity and the function of the cellular components can be a more important determinant of the clinical manifestations of atheroslcerosis than the degree of stenosis of the arterial lesions. Although many factors may initiate atherosclerosis, the process ultimately involves an inflammatory state in which macrophages and T-lymphocytes play a major role[22,29,38,43].

There is a growing amount of evidence to support an association between infection and atherosclerosis, with the first suggestion of a link being made by Osler early in this century. Since Fabricant *et al.* (1978) first reported that herpesviruses infection is as a risk factor for atherosclerosis, a number of seroepidemiological studies have shown a positive relationship between cytomegalovirus infection and atherosclerosis[4,16,34]. The results of *in situ* hybridization and PCR study also demonstrated a positive association between cytomegalovirus infection and atherosclerosis[12].

As mentioned above, vascular inflammation and a chronic degenerative process are prerequisite for atherosclerosis. NF-κB, a ubiquitous transcription factor of particular importance in immune and inflammatory responses, increases the expression of the genes for many cytokines, enzymes, and adhesion molecules in chronic inflammatory diseases. This increased expression is reflected by an increased amount of nitric oxide, which has a cytotoxic effect on vascular tissue. Cyclooxygenase-2 (COX-2) another inducible enzyme regulated by NF-κB, is responsible for the increased production of prostaglandins (PG) and thromboxane (TXA) in inflammatory diseases[32]. Induced COX-2 expression in monocytes-macrophages and fibroblasts results in increased synthesis of PGE2 and TXA2, which mediate inflammatory change, vasoconstriction, and platelet aggregation. The increased synthesis of COX-2 is considered to play an important role in inflammation and tissue injury. COX-2, which is induced in many cell types in response to cytokines, metabolizes membrane phospholipid arachidonic acid and plays a role in the expression of an inflammatory mediator in heart failure[48]. Gelatinase-B also known as 92-kDa gelatinase or MMP-9, may contribute importantly to the instability of human atherosclerotic plaques. The regulation of transcription of MMP-9 depends in part on a NF-κB element in its promoter sequence. This transcription factor is known to be regulated by oxidative stress and may link the accumulation of oxidized lipoproteins in the intima to expression of this particular protease[1].

This study was designed to test the hypothesis that the immunoreactivity for CMV late antigen and inflammatory mediators such as COX-2 and MMP-9 will be colocalized to inflammatory cells in atheromatous plaque if cytomegalovirus has some pathogenetic role on atherosclerotic diseases. That is, if the vascular infection of cytomegalovirus could induce a chronic inflammatory reaction in host vascular tissue and activate inflammatory cells, some inflammatory mediators such as COX-2 and MMP-9 may exhibit increased expression surrounding macrophages, which have key role in atherosclerosis, infected with cytomegalovirus. The results of this study demonstrated that COX-2 and MMP-9 were colocalized to the inflammatory infiltrates of diseased tissues, particularly within cytomegalovirus-stained macrophages/monocytes.

Elevated lipids may play a role in initiating atherosclerotic lesion development by stimulating inflammatory cytokine production, which may in turn initiate the inflammatory process. Inflammatory cytokines were localized to macrophages and endothelial cells overlying atherosclerotic

lesions. Minimally oxidized LDL and beta-VLDL upregulate inflammatory cytokines, thereby attracting more leukocytes to develop atherosclerotic plaque, beginning the cycle of inflammation and intimal development[5,47]. As well, oxidized LDL and vascular endothelial growth factor (VEGF) accumulated in human atherosclerotic lesions induces the production of macrophage VEGF[37]. Also, VEGF upregulates the expression of matrix metalloproteinases in vascular smooth muscle cells[46]. Similar events may occur in the adventitia during atherosclerotic development where the adventitial vasa vasorum endothelial cells may be affected by elevated lipids and express adhesion molecule early in the atherosclerotic process, thus contributing to the invasion of inflammatory cells into the adventitia as well[5,47,49]. Experimental hypercholesterolemia may induce the upregulation of specific cell-surface receptors in the adventitial vasa vasorum, which then predisposes them to proliferate in response to growth factors and an increase in the vessel wall thickness[11,21,25]. Several experimental studies have shown that in response to and increased presence of mildly oxidized lipoproteins in the outer media[23], which have been shown to be chemotactic for monocytes[36], an increased number of monocytes can enter the intima and adventitia under hypercholesterolemic conditions[27].

This study documents that there was significant expression of COX-2 and MMP-9 in atherosclerosis aorta and coronary artery, but very little in control tissues. Immunoreactivity for COX-2 and MMP-9 were colocalized to the inflammatory infiltrates, principally macrophages/foam cells in atherosclerotic intima, plaque itself, and vascular smooth muscle cells. In addition, these expressions were evident in the medial smooth muscle cells. These results suggest that COX-2 and MMP-9 have pathobiological roles in cytomegalovirus-infected atheroslcerosis as the inflammatory mediator or its product, which may regulate cellular activation and reorganize extracellular matrix.

Macrophages are known to play an important role in regulating the turnover of extracellular matrix (ECM) in both normal and pathologic conditions through the secretion of proteases, including MMPs, protease inhibitors, and cytokines. MMP expression in macrophages is dependent on prostaglandin $E_2$ ($PGE_2$)[8]. $PGE_2$ synthesized from arachidonic acid, and cyclooxygenase (COX) is the rate-limiting enzyme in this pathway. The activation of macrophages has been previously correlated with the induction of COX-2[3]. Macrophages expressing COX-2 are known to produce eicosanoids that have proinflammatory effects, increasing vascular permeability, promoting chemotaxis, and favoring cell proliferation and cholesterol ester retention[17,19].

Recent evidence is of particular relevance to the role of COX-2 in inflammation and atherosclerosis. COX-2 in activated human monocytes may be able to generate the prostaglandin, 8-epi-$PGF_{2\alpha}$, which is mitogenic leading to cellular proliferation, and vasoconstrictive leading to vasoconstriction, and thus may play a role in the genesis of atherosclerosis[13,35,42]. In this study, the immunolocalization study confirmed the increased expression of COX-2 in atherosclerotic plaque, especially in macrophages.

Increased levels of several MMPs, including stromelysin, interstitial collagenase, and gelatinase A and B, show increased expression and/or activation in atherosclerotic plaques [14,15,18,26,50]. Furthermore, MMP-9 expression and MMP-2 expression and activation are positively correlated with lesion severity, consistent with a pathogenetic role in the late disease process[50]. The activation of MMPs and their proteolytic potential is tightly controlled by endogenous tissue inhibitors of matrix metalloproteinases (TIMPs)[6,7]. This study showed coexpression of MMP-9 and COX-2 in macrophages and smooth muscle cells in intima and media, suggesting that inflammatory activation of MMP-9 as well as COX-2, may contribute to the enhanced local matrix degradation in atherosclerotic plaques.

The present study demonstrated that immunoreactivity for MMP-9 and COX-2 were noted in the intimal smooth muscle cells. Vascular adhesion molecules like intercellular adhesion molecule-1 (ICAM-1) have been characterized in the endothelial cells of the vasa vasorum[41]. These molecules aid in the recruitment of inflammatory cells to the aorta and stabilize local T-cell receptor function[20]. This finding suggested that inflammatory reaction was evident coupling with intimal hyperplasia. Linton *et al.*[28] demonstrated that COX-2 expression was also found in aortic lesions of apoE-deficient mice in fatty streaks and complex atherosclerotic lesion areas using paraffin sections. These results were concordant with this study.

In conclusion, this study demonstrated the presence of both COX-2 and MMP-9 in atherosclerotic lesions of aorta and coronary artery. These findings support the hypothesis that COX-2 and MMP-9 may interact and play a role in this disorder.

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