

Coexpression of Cyclooxygenase-2 and Matrix Metalloproteinases in Human Aortic Atherosclerotic Lesions

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Abstract

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 aimed to investigate atherosclerosis in human aortas for in situ tissue distribution of COX-2, MMPs including MMP-9 and membrane type 1 MMP (MT1-MMP), and tissue inhibitor of metalloproteinase-2 (TIMP-2). Immunohistochemical studies were performed on atherosclerotic lesions of aortas from patients with aortic aneurysms (n=4) and dissections (n=3) by using antibodies to COX-2, MMP-9, MT1-MMP, and TIMP-2. Control tissues were obtained from traumatically dissected aortas (n=2). All specimens from diseased aortas had atherosclerotic lesions ranging from fatty streak to atheromatous plaques. In control, there was no expression of COX-2, MMP-9, and MT1-MMP in all aortic layers. Immunoreactivity for COX-2 was predominantly noted in macrophages and smooth muscle cells (SMCs) of the intima including atherosclerotic plaque itself and the medial layer of the plaque base, as well as in SMCs and endothelial lining of the vasa vasorum in the adventitia. Immunoreactivity for MMP-9 and MT1-MMP was found in the same distribution as that of COX-2. Additionally, the expression of TIMP-2 increased in relation to MMP-9 expression. This study demonstrates that COX-2 is coexpressed with MMP-9 and MT1-MMP, not only by macrophages and SMCs in atherosclerotic lesions, but also in endothelial lining of the vasa vasorum of human aortas. Thus, vascular inflammatory reactions may influence extracellular matrix remodeling by coactivation of MMPs in the development of atherosclerosis and, in turn, the progression of disease.

Key Words: Atherosclerosis, cyclooxygenase-2, matrix metalloproteinase

INTRODUCTION

Atherosclerosis is currently considered to be an exaggerated response of the vessel wall to injury characterized by inflammation and fibrocellular proliferation rather than a degenerative disease due to

hemodynamic loading.^{1,2} This view is supported by the demonstration of abundant macrophages and T lymphocytes in atherosclerotic plaque, as well as studies examining markers of inflammation (eg. c-reactive protein).³⁻⁵ Plaque rupture leading to thrombosis is the key event in myocardial infarction and it has been shown to be related to increased inflammation within the plaque, rather than plaque morphology or degree of vessel stenosis.⁶ Chronic inflammatory lesions are often associated with a significant destruction of connective tissue. The association of the monocyte/macrophage with lesions where there is the destruction of connective tissue in atherosclerotic tissue favors their participation in the breakdown of collagen.

The production of matrix metalloproteinases (MMPs) by human monocytes has been shown to occur through a prostaglandin (PG) E₂-cAMP dependent pathway.⁷⁻⁹ Signaling through this pathway involves

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the modulation of prostaglandin H synthase, that is, cyclooxygenase (COX).¹⁰ Two COX isoforms 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 activators 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 PGE₂ has been shown to be involved in the signal transduction pathway leading to the production of MMPs by these cells.¹¹

In view of the interactions of COX-2 and MMPs, we hypothesized that the inflammation via COX-2 and the production of MMPs might be involved in the development of atherosclerosis in human aortas. The aim of this study was to determine the cellular location of COX-2, and to investigate for codistribution of COX-2 and MMPs.

MATERIALS AND METHODS

Tissue preparation and histologic examination

The study population consisted of 7 patients (6 men and 1 woman; range of age, 57–74 years; mean age, 67 years) with atherosclerotic aortic aneurysm (4 patients) and dissection (3 patients) of aorta, who were all referred to Severance Hospital (Seoul, Korea) for evaluation and surgical treatment between 1990 and 1998. For control studies, aorta specimens, from which atherosclerotic lesions including fatty streak and plaque were excluded, were obtained from 2 patients (ages, 20 and 26 years) who were surgically treated for traumatic aortic dissection.

Immediately following removal of aortic segments, each segment was fixed with buffered 10% formalin to maintain morphologic integrity. To preserve the integrity of the adventitia and perivascular tissues, aortic specimens were carefully removed in a segment along with adjacent tissues and rinsed with PBS (phosphate buffered saline). Each segment was embedded in paraffin and cut in 5 μ m 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 mor-

phological 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.

Immunohistochemistry

Immunohistochemical studies were made using goat polyclonal, primary antibodies against COX-2, MMP-9, MT1-MMP, and TIMP-2 of human origin (Santa-Cruz Biotechnology Inc., Santa Cruz, CA, USA). Peroxidase-conjugated secondary antibodies were used with these primary antibodies.

Paraffin sections (5 μ m) were made and transferred to glass slides. The paraffin sections were deparaffinized and rehydrated through the following solutions: xylene 3 times for 3 minutes in each, and serially 100%, 95%, 80%, and 75% ethanol for 3 minutes in each. The sections were then treated with 0.3% H₂O₂ in methanol for 5 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 (800 W) to retrieve antigen sites, and cooled at room temperature for 20 minutes, followed by washing in PBS (pH=7.2–7.4). 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. The sections were then processed by the streptavidin-biotin-peroxidase complex method using the LSAB plus kit (DAKO Inc., Carpinteria, CA, USA), and stable DAB solution (3,3'-diaminobenzidine tetrahydrochloride, Research Genetics Inc., Huntsville, AL, USA) was used as a chromogen. Mayer's hematoxylin was used as a counterstain, and sections were dehydrated, cleared, and mounted.

RESULTS

Histologic analysis

Sections of aorta taken from traumatic dissections showed no significant histological evidence of atherosclerosis except minimal intimal thickening and

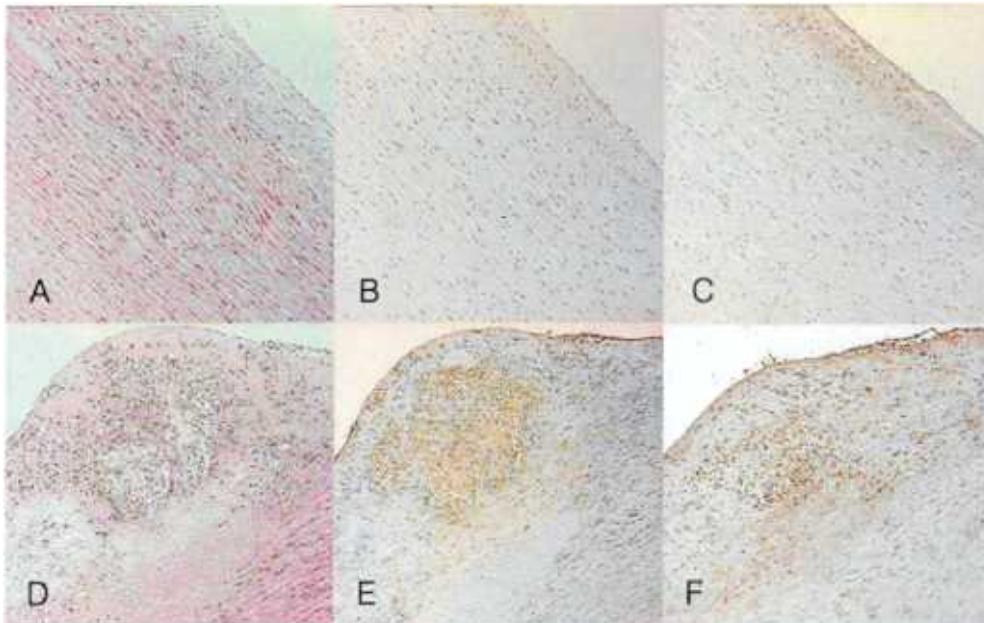


Fig. 1. Hematoxylin & eosin stain, immunohistochemistry for COX-2 and MMP-9 of control section (panel A-C, respectively) and atherosclerotic section (panel D-F, respectively; final magnification, $\times 100$). Sections of aorta taken from traumatic dissections (panel A) showed no significant histological evidence of atherosclerosis and normal patterns of elastic media. Compared with controls, there was prominent inflammatory infiltration with mononuclear cells and foam cells in atherosclerotic plaque, especially in the central core (panel D). In nonatherosclerotic section (panel B-C), immunoreactivity for COX-2 and MMP-9 were not shown. Atherosclerotic lesion (panel E-F) demonstrated plaque with a lipid core, which showed strong immunoreactivity for COX-2 and MMP-9.

normal patterns of elastic media. However, the present study could not analyze the control adventitial layer because traumatic injury had occurred at the adventitia (Fig. 1A).

Compared with controls, atherosclerotic lesions that ranged from fatty streak to complicated atherosclerotic plaque were noted in all patients with atherosclerotic aortic diseases. Atherosclerotic lesions were patchily distributed along the upper base of the dissecting plane, especially in specimens from aortic dissections. There was also prominent inflammatory infiltration with mononuclear cells and foam cells in atherosclerotic plaque, especially in the central core (Fig. 1D), and to a lesser extent in the inner media and adventitia (data not shown).

Immunohistochemistry

Sections from control specimens showed little immunoreactivity for COX-2, MMP-9 and TIMP-2 in the minimal thickened intima, and no immunore-

activity in the media (Fig. 1, B and C).

In atherosclerotic sections (plaque with central core), immunoreactivity for COX-2 and MMP-9 was evident in all cases of aortic atherosclerosis along with plaques, mainly in macrophages/foam cells, intimal and medial smooth muscle cells, and endothelial cells of the intima (Fig. 1E-F, and 2A-C). Immunoreactive patterns between COX-2 and MMP-9 were similar. Furthermore, the immunoreactivity for both of them was also noted in the outer media and adventitia, especially the endothelial lining of neovascularized vasa vasorum (Fig. 2, D and E). Immunoreactivity for MT1-MMP and TIMP-2 were found in a similar pattern and distribution with COX-2 and MMP-9 (Fig. 2, B, C, E, and F). In fatty streak, immunoreactive patterns for COX-2, MMPs, and TIMP-2 were similar, although immunoreactivity was less prominent (figures were not shown).

On histopathologic analysis coupling the adventitia inflammatory reaction with the intimal reaction, SMCs, inflammatory cells and endothelial cells of neo

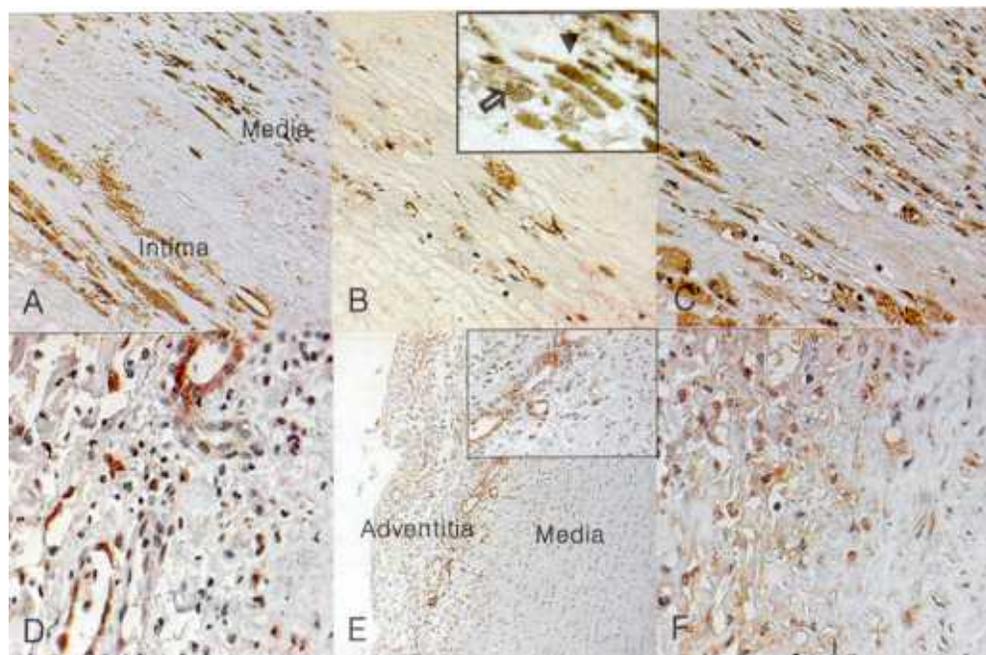


Fig. 2. Immunoreactivity for COX-2, MT1-MMP, and TIMP-2 in the medio-intimal (upper panels; A-C) and medio-adventitial junction (lower panels; D-F) of atherosclerotic lesions. Note the similar distribution of COX-2, MT1-MMP and TIMP-2 reactivity in the medio-intimal junction, especially in macrophage (open arrow in box of panel B) and SMCs (arrow in box of panel B). In the medio-adventitial junction, immunoreactivity for COX-2 and MT1-MMP was codistributed, especially in endothelial lining of the neovascularized vasa (box of panel E). Final magnification, $100\times$ (E), $400\times$ (A-D, F, and box in E), and $1000\times$ (box in B).

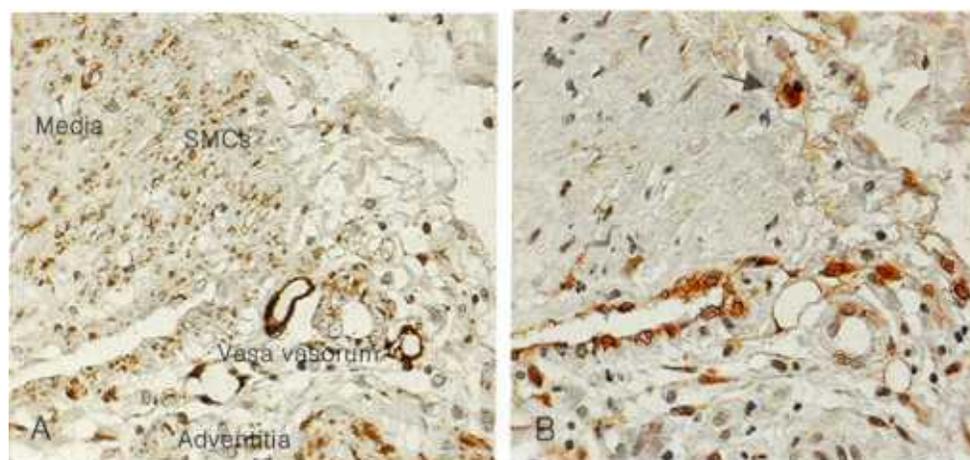


Fig. 3. Differential immunoreactivity of COX-2 and MT1-MMP for cellular components of the medio-adventitia. Whereas MT1-MMP (A) was predominantly expressed in SMCs of the media adjacent to the adventitia and the vasa, COX-2 (B) was strongly expressed in macrophages and endothelial lining of the vasa. Final magnification, $\times 400$.

vascularized vasa vasorum of the outer media revealed strong immunoreactivity for COX-2 and MT1-MMP.

However, there was somewhat differential immunoreactivity of COX-2 and MT1-MMP for cellular com-

ponents of the medio-adventitia. While MT1-MMP expressed predominantly in medial smooth muscle cells and to a lesser extent in endothelial lining of the vasa, COX-2 expressed strongly in macrophages and endothelial lining of the vasa (Fig. 3).

DISCUSSION

This study documents that there was significant expression of COX-2 and MMPs in atherosclerotic aortic tissues, but very little in control aortic tissues. Immunoreactivity for COX-2 and MMPs were colocalized to the inflammatory infiltrates, principally macrophages/foam cells in atherosclerotic intima, plaque itself, and vascular SMCs. In addition, these expressions were evident in the medial SMCs and endothelial cells of the vasa vasorum in adventitia. These results suggest that COX-2 and MMPs including MT1-MMP have pathobiological roles in atherosclerosis 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 E₂ (PGE₂).¹² PGE₂ is 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-2.¹³ 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.^{14,15}

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-PGF₂ α , which is mitogenic leading to cellular proliferation, and vasoconstrictive leading to vasoconstriction, and thus may play a role in the genesis of atherosclerosis.¹⁶⁻¹⁸ Our immunolocalization study confirmed the increased expression of COX-2 in aortic atherosclerotic plaque, especially in macrophages.

Increased levels of several MMPs, including stro-

melysin, interstitial collagenase, and gelatinase A and B, show increased expression and/or activation in atherosclerotic plaques.¹⁹⁻²³ Furthermore, MMP-9 expression and MMP-2 expression and activation are positively correlated with lesion severity, consistent with a pathogenetic role late in the disease process.²³ The activation of MMPs and their proteolytic potential is tightly controlled by endogenous tissue inhibitors of matrix metalloproteinases (TIMPs).^{24,25} Four members of the TIMP family are known to date, among which only TIMP-1, -2, and 3 have been characterized in vascular tissue.^{19, 26-29} Studies with isolated smooth muscle cells suggest that both TIMP-1 and 2 secretion is constitutive, and in one immunohistochemical study of human arteries, TIMP-1 and 2 were identified at similar levels in atherosclerotic and normal tissue.^{19,27,29} However, in another study, immunoreactive TIMP-1 was detected at apparently higher levels in human atherosclerotic and late restenotic carotid arteries compared with normal arteries.²⁸ These indicate that the data for TIMPs are conflicting in contrast to the consensus of evidence for increased activity of MMPs in atherosclerosis. Galis et al reported the increase of TIMP-1 secretion and a parallel increase in gelatinase expression.²¹ In this study, TIMP-1 immunoreactivity was noted at the area of MMP-9 expression, which suggests that in atherosclerosis, the increase expression of TIMPs counterbalances the activity of the MMPs.

MT1-MMP is a 64-kDa protein that contains a single transmembrane domain with the catalytic site positioned on the exterior surface of the cell.³⁰ MT1-MMP belongs to a family of cell-bound proteins that specifically activate pro-MMP-2, which, unlike other MMPs, is not activated by plasmin proteolysis by MT1-MMP.³¹⁻³³ Recent studies have suggested a unique mechanism by which MT1-MMP activated pro-MMP-2.^{34,35} MT1-MMP-induced activation of pro-MMP-2 appears to involve the formation of a complex with TIMP-2, which in turn can form a ternary complex of MT1-MMP/TIMP-2/pro-MMP-2. Tripathi et al demonstrated immunohistochemical evidence for TIMP-2 expression in macrophage-rich regions of human atheroma using in situ zymography.³⁶ Pro-MMP-9, pro-gelatinase B, as well as pro-MMP-2 is also activated to MMP-9 via MT1-MMP.³⁷ We demonstrated immunohistochemical evidence for TIMP-2 expression in macrophage-rich regions of human aortic atherosclerotic plaque, which

is coexpressed with MMP-9. Our study also showed coexpression of MT1-MMP and COX-2 in macrophages and SMCs in intima and media, suggesting that inflammatory activation of MT1-MMP as well as COX-2, may contribute to the enhanced local matrix degradation in atherosclerotic plaques. This is supported by previous reports of the upregulation of MT1-MMP mRNA by inflammatory cytokines like TNF- α and IL-1 β .^{36,38}

Interestingly, the present study demonstrated that immunoreactivity for MMP-9, COX-2, and MT1-MMP were noted in the endothelial lining of vasa vasorum in the adventitia and outer medial smooth muscle cells. Vascular adhesion molecules like intercellular adhesion molecule-1 (ICAM-1) have been characterized in the endothelial cells of the vasa vasorum.³⁹ These molecules aid in the recruitment of inflammatory cells to the aorta and stabilize local T-cell receptor function.⁴⁰ This finding suggested that adventitial inflammatory reaction was evident coupling with intimal hyperplasia. Although the present study did not evaluate enzymatic expression using RT-PCR and zymography from the fresh tissues, the antigen retrieval method was used for immunohistochemistry of COX-2, MMPs, and TIMP-2 with limited specimens. However, the immunoreactivity for COX-2, MMPs, and TIMP-2 were seen in macrophage and smooth muscle cells exclusively in atherosclerotic lesions. Linton et al. 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.⁴¹ These results were concordant with our study. In future, more precise methods and experimental studies to evaluate this hypothesis and will be needed and applied in therapeutic trials.

In conclusion, this study demonstrated the presence of both COX-2 and MMPs in aortic atherosclerotic lesions, and these findings support the hypothesis that COX-2 and MMPs may interact and play a role in this disorder.

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