Systemic Arterial Expression of Matrix Metalloproteinases 2 and 9 in Acute Kawasaki Disease

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Objective—Coronary artery aneurysms are the major complication of Kawasaki disease (KD). Matrix metalloproteinases (MMPs) regulate remodeling and degradation of the extracellular matrix. We hypothesized that MMP-9 expression is increased in acute KD aneurysms when compared with KD nonaneurysmal arteries and arteries from control children.

Methods and Results—MMP-2, MMP-9, tissue inhibitor of metalloproteinase (TIMP)-1, and TIMP-2 were immunolocalized in coronary arteries from children with fatal acute KD and controls. In KD coronary aneurysms, MMP-2 expression was prominent in the thickened neointima and in endothelial cells of new capillaries in areas of angiogenesis. MMP-9 was absent in control coronary arteries but was expressed in coronary artery aneurysms, nonaneurysmal coronary and noncoronary arteries, and cardiac nerves in acute KD, without an increase in TIMP-1 expression.

Conclusions—MMP-2 likely participates in remodeling of the arterial wall in acute KD, particularly in the processes of neointimal proliferation and angiogenesis. MMP-9 may play a role in the development of coronary aneurysms, but its expression is not confined to aneurysmal arteries. Systemic arterial expression of MMP-9 in acute KD, even in the absence of inflammatory changes in the vessel, suggests induction by a circulating factor, or possibly by an infectious agent with tropism for arterial tissue. (Arterioscler Thromb Vasc Biol. 2003;23:●●●●●●●●)

Key Words: Kawasaki disease ▪ matrix metalloproteinases ▪ coronary artery ▪ aneurysm

Kawasaki disease (KD) is a potentially fatal, acute vasculitis of childhood, which has surpassed acute rheumatic fever as the most common cause of acquired heart disease in children in the United States and other developed nations.1,2 Significant cardiovascular sequelae can complicate disease in children in the United States and other developed nations.1,2 Significant cardiovascular sequelae can complicate disease in children in the United States and other developed nations.1,2 Significant cardiovascular sequelae can complicate disease in children in the United States and other developed nations.1,2 Significant cardiovascular sequelae can complicate disease in children in the United States and other developed nations.1,2 Significant cardiovascular sequelae can complicate disease in children in the United States and other developed nations.1,2 Significant cardiovascular sequelae can complicate disease in children in the United States and other developed nations.1,2 Significant 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immunoglobulin binding was blocked with 4% normal horse serum in PBS for 30 minutes. Sections were incubated overnight at 4°C with mouse anti-human MMP-2 monoclonal antibody (1:250 in PBS) (MMP-2, AB-4, Neomarkers Inc), mouse anti-human MMP-9 monoclonal antibody (1:100 in PBS) (MMP-9, AB-3, Oncogene Research Products, distributed through Calbiochem), mouse anti-human TIMP-1 (1:50 in PBS) (TIMP-1 Ab-2, clone 102D1, Neomarkers), or mouse anti-human TIMP-2 (1:250 in PBS) (TIMP-2 AB-5, clone 3A4, Neomarkers). Both MMP antibodies react with the pro- and active forms of their respective enzymes. After the tissues were washed in PBS, staining was detected with a biotinylated horse anti-mouse IgG secondary antibody (Vector Laboratories Inc) and an avidin-biotin–horseradish peroxidase system (Vectastain Elite ABC system, Vector Laboratories Inc). With diaminobenzidine tetrahydrochloride as a reaction product, positive cells stained brown. Sections were counterstained with Gill's hematoxylin (Vector Laboratories Inc). In noninflamed KD and normal coronary arteries, the endothelial cell layer (intima), media, and adventitia were each graded for expression of MMP-2 and MMP-9 as described in the following section. In all aneurysmal and nonaneurysmal but inflamed coronary arteries, disruption of the internal elastic laminas generally obscured the normal boundaries of intima and media. To reflect this morphology more accurately, grading of the myointimal and adventitial layers was performed. Immunological staining was graded and scored as follows: 0, no evidence of staining (eg, Figure 1D, all layers); 1, mild focal vascular staining (eg, Figure 1F, adventitial layer); 2, moderate vascular staining (eg, Figure 1F, myointimal layer); and 3, marked vascular staining (eg, Figure 1C, myointimal layer).

Positive control tissues for immunohistochemistry included normal spleen for MMP-9, normal blood vessel for MMP-2 and TIMP-2 (both of these molecules are normally expressed by smooth muscle cells), and breast cancer tissue (Neomarkers) for TIMP-1. Negative controls included sections in which the primary antibody was omitted.

### Statistical Analysis

Wilcoxon’s sum of ranks test was used to compare the grade of immunohistochemical staining of corresponding layers of control and KD coronary arteries by using the antibodies. Wilcoxon’s signed rank test was used to compare the grade of staining of the KD CAAs with their matched (in the same patient) nonaneurysmal or noninflamed coronary artery. A value of \( P < 0.05 \) was considered significant.

### Results

#### Histopathology of CAAs in KD

KD CAAs demonstrated a characteristic pathologic appearance, with disruption of the distinct trilaminar structure of the arterial wall (intima, media, and adventitia) and marked transmural infiltration of inflammatory cells. Von Gieson’s elastic stain of a section of control coronary artery demonstrated intact internal and external laminas (Figure 1A), whereas marked fragmentation of the elastic laminas was seen in CAAs of patients with acute, fatal KD (Figures 1B, 1C, and 2G). Marked thinning of the vascular media and thickening of the vascular intima were evident in CAAs, as previously described in KD.5

#### MMP-2 Expression in KD and Control Coronary Arteries and Myocardium

In all control coronary arteries, MMP-2 expression was identified in smooth muscle cells within the media, which was separated from the intima by an intact internal elastic lamina, and in the single layer of endothelial cells within the intima (Figure 2A). Similar semiquantitative MMP-2 expres-
MMP-9 Expression in KD and Control Coronary Arteries and Myocardium

MMP-9 expression was not demonstrated in control coronary arteries (Figures 2D and 3C). However, MMP-9 was expressed in KD CAAs (Figure 2F), nonaneurysmal coronary arteries (Figures 2E and 3D), and in noninflamed coronaries. The myointimal layers of CAAs and nonaneurysmal KD arteries both demonstrated a statistically significant increase in MMP-9 staining when compared with either intima or media of controls ($P<0.05$), nonaneurysmal KD arteries vs controls ($P<0.05$ for nonaneurysmal KD CA arteries vs controls). In addition, noninflamed KD coronary intima and media showed a statistically...
significant increase in MMP-9 staining when compared with controls ($P<0.05$ for both endothelial cells and media). MMP-9 staining was present in smooth muscle cells and infiltrating mononuclear cells. KD patients also had a significant increase in MMP-9 staining in myocardium compared with controls ($P=0.01$; Figure 3C and 3D). In KD patient 10, MMP-9 expression was not observed in the coronary artery or myocardium; this patient had received aggressive immunosuppressive therapy with high-dose corticosteroids and methotrexate. To determine whether MMP-9 was expressed in

**Figure 2.** MMP-2 and MMP-9 in coronary arteries of KD patients and controls. A–C, G, and H, Immunohistochemical stains for MMP-2. A, Demonstration of MMP-2 staining of intimal endothelium and medial smooth muscle cells in control coronary artery. In B, MMP-2 expression is observed in nonaneurysmal coronary artery from a KD patient; there is increased expression in adventitia and less expression in media when compared with control vessel. In C, MMP-2 expression is seen in a CAA from a KD patient. There is intense staining of myointima (MI) and of endothelium of new blood vessels arising in this area (H; black arrows). Breaks in internal elastic membrane are evident in G (yellow arrows indicate intact membrane on either side of a break); MMP-2–positive cells appear to be migrating through breaks in the membrane. D–F, Immunohistochemical stains for MMP-9. D, Demonstration of absence of MMP-9 expression in the same control coronary artery in A. E, Demonstration of MMP-9 expression in the same KD nonaneurysmal coronary artery shown in B. MMP-9 expression is observed diffusely throughout the arterial wall but is particularly prominent in myointima. F, MMP-9 expression in the same KD CAA shown in C. MMP-9 expression is seen diffusely throughout the vessel wall but is particularly notable in myointima in this patient. L indicates lumen; I, intima; M, media; MI, myointima. A–F taken with a ×20 objective; panels G, H, with a ×40 objective.

**Figure 3.** MMP-9 in cardiac peripheral nerves, myocardium, and adventitia from KD patients and controls. A, Control peripheral nerve showing absence of MMP-9 staining. B, KD peripheral nerve showing strong MMP-9 staining. C, Control myocardium and adventitia showing staining of myocardium (black arrow) and no staining of peripheral nerve or nearby coronary artery (green arrow). D, KD myocardium and adventitia showing staining of myocardium (black arrow) and staining of nearby nonaneurysmal coronary artery. A–D taken with a ×20 objective.
noncoronary arteries in KD, sections of mesenteric, renal, and pancreatic arteries that were available from 8 of the 11 KD patients were examined; MMP-9 staining was also observed in the wall of these noncoronary arteries from 6 of the 8 KD patients. A marked increase in MMP-9 staining of peripheral nerves in the KD coronary artery adventitia when compared with nerves in control coronary artery adventitia was also noted ($P=0.002$; Figure 3A, 3B, and 3C).

**Discussion**

MMP-2 and MMP-9 appear to play a role in the formation of abdominal aortic aneurysms in adults and may be involved in aneurysmal dilatation of arteries in other diseases such as KD. MMP-2, which is expressed in the intima and media of normal arteries, was also prominent in the myointima of acute KD CAAs and was identified in medial smooth muscle cells that appeared to be migrating into the intima through breaks in the internal elastic lamina. Notably, MMP-2 expression was observed in endothelial cells of new capillaries in areas of angiogenesis in the myointima and adventitia of KD CAAs. Angiogenesis has been reported in CAAs from KD children who died years after onset, but has not been reported previously in coronary aneurysms in the acute phase; this interesting finding deserves further study. MMP-2 expression paralleled MMP-2 expression in acute KD. Increased expression of both molecules was observed in the adventitia of KD CAAs and was identified in medial smooth muscle cells for example, that an MMP-9–positive cell in 1 section and a CD68-positive cell on an adjacent section were definitely the same cell. We therefore reported cell types positive for the same protein. Thus, our results include detection of both pro- and active forms of MMP-2 and MMP-9. Zymography, which requires fresh or snap-frozen tissue samples, is the optimal technique to demonstrate the presence of the active form of MMPs. Obtaining fresh or frozen arterial tissue samples from acute KD fatalities has proven problematic; tissue is generally placed in formalin as routine procedure at autopsy. Despite acquiring a large collection of KD autopsy specimens from throughout the United States and Japan, we have not been able to obtain nonformalin-fixed tissue that could be used in zymography. Additionally, we were not able to confirm the cell types producing the MMPs and TIMPs by double staining, because immunofluorescence studies on archival formalin-fixed, paraffin-embedded tissues are difficult to perform. Staining of sequential sections for cell markers and MMPs or TIMPs was not useful because of a lack of specific landmarks in the inflamed vascular wall. After disruption of the normal architecture of the arterial wall and infiltration by copious inflammatory cells, it was very difficult to be certain, for example, that an MMP-9–positive cell in 1 section and a CD68-positive cell on an adjacent section were definitely the same cell. We therefore reported cell types positive for the MMPs and TIMPs based on morphological appearance.

Autopsy studies of aneurysms in the acute phase of KD generally reveal marked inflammatory cell infiltration in the aneurysm. It is possible that invading inflammatory cells in the KD aneurysm result in the production of more active MMP-9 than in noninflamed vessels, leading to more dilatation or aneurysm formation in the segments of coronary artery with the most severe degree of inflammatory cell infiltration.

Widespread expression of MMP-9 in arterial tissue in acute KD, even in the absence of inflammatory or aneurysmal changes in the vessel, suggests induction by factors circulating in the bloodstream. Tumor necrosis factor-α has been shown to induce MMP-9 in monocytes and circulating levels have been reported to be elevated in acute KD.
However, MMP-9 was not detected in coronary arteries of 3 controls who died of bacterial meningitis and Gram-negative sepsis, conditions in which tumor necrosis factor-α is also likely to circulate. Interestingly, MMP-9 was detected prominently in peripheral nerves in KD coronary artery adventitia. Nerve growth factor induces MMP-9 expression in vascular smooth muscle cells, and dramatically increased levels of nerve growth factor have been reported in sera in the acute phase of KD; this possible relationship deserves further study. It is also possible that MMP-9 is induced by the infectious etiologic agent of KD. Clinical and epidemiologic features of KD support an infectious cause; however, the etiologic agent remains unknown. Epstein-Barr virus, influenza A virus, human T-cell leukemia virus type 1, respiratory syncytial virus, and mycobacteria induce expression of MMP-9 in a variety of human and animal cell lines. Thus, expression of MMP-9 in acute KD arteries may result from induction by an infectious agent with tropism for arterial tissue.

In summary, systemic arterial expression of MMP-2 and MMP-9 was identified in acute KD. In CAA, MMP-2 was prominent in thickened neointima and endothelial cells of new capillaries in areas of angiogenesis in the neointima and adventitia. MMP-9 was detected in arteries, nerves, and myocardium in acute KD and was not accompanied by increased expression of TIMP-1. Widespread expression of MMP-9, even in the absence of inflammatory changes in the vessel wall, suggests induction by factors circulating in the bloodstream, or possibly by an infectious agent with tropism for arterial tissue.

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References

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