awasaki disease (KD) is characterized by a systemic arteritis that causes structural weakening of vessel walls, which can induce coronary aneurysms.\(^1\)-\(^3\) Following the destruction of vessel walls, intimal hypertrophy develops and often causes coronary stenotic lesions.\(^3\) KD is thus a major cause of ischemic heart disease in childhood.

The remodeling of the extracellular matrix (ECM) is a key histological feature of the vascular lesions of KD because ECM molecules constitute the basement membrane that contributes structural support to the vasculature.

The synthesis and degradation of the ECM-constituted basement membrane are modulated mainly by interactions between matrix metalloproteinases (MMPs) and cognate tissue inhibitors of MMPs (TIMPs).\(^4\)-\(^6\) Gelatinases A (MMP-2) and B (MMP-9) metabolize native type IV collagen, a major matrix constituent of the basement membrane. MMP-2 is constitutively expressed in a variety of cells, and MMP-9 is responsive to various cytokines and is produced mainly by connective tissue cells such as fibroblasts and macrophages.\(^7\)-\(^11\) Consequently, MMP-2 and -9 are implicated in numerous pathological conditions, including atherosclerosis, inflammation, tumor metastasis, and KD.\(^12\)-\(^14\) The regulatory mechanisms of MMPs and TIMPs in KD are still unclear. The present study focused on endothelial cells (ECs) as producers of MMP-9 and TIMP-1, and examined the regulatory mechanisms of MMP-9 by ECs through cytokines that are increased in KD.

**Methods**

**Subjects**

We enrolled 30 children with acute KD: 19 boys and 11 girls, aged 3 months to 5 years. No coronary sequelae was observed in these patients, except for transient dilatation of the left coronary artery in 1. Non-febrile controls comprised 15 children who underwent catheter intervention for small patent ductus arteriosus or preoperative catheterization for small ventricular septal defect with right coronary cusp deformity: 9 boys and 6 girls, aged 5 months to 3 years.
controls were 25 children with febrile diseases, including measles, influenza, and streptococcal infection: 12 boys and 13 girls, aged 10 months to 4 years.

Sample Collection
Blood samples consisted of plasma or serum samples from the subjects; samples were collected in commercially obtained EDTA-2K or plain tubes and were stored at ~80°C until use. Peripheral blood mononuclear cells (PBMCs) were obtained from EDTA-2Na whole blood using Ficoll-Paque reagent. Serial samples were obtained in the KD group during the clinical phases; phase I: within 7 days of the onset of the disease before intravenous high-dose γ-globulin therapy (IVIG); phase II: just after IVIG; and phase III: at 1 month from the onset of the disease; ND, not done; H, healthy controls.

Culture of ECs
Cells lines established from human umbilical vein ECs (HUVECs) were maintained in Dulbecco’s modified Eagle’s medium supplemented with 5% fetal bovine serum (FBS; GibcoBRL, Grand Island, NY, USA), 2 mmol/L L-glutamine, kanamycin (50 mg/mL), in 5% CO₂ atmosphere at 37°C. HUVECs were precultured in non-serum medium for 24 h prior to each study.

Preparation of Cytokines and Specific Antibodies
Recombinant human interferon (IFN)-γ; Kyowa-Hakko, Tokyo, Japan), interleukin (IL)-1β, IL-6, and tumor necrosis factor (TNF)-α; R&D System Inc, Minneapolis, MN, USA) were prepared for the study. Specific antibodies for each cytokine were purchased from R&D System.

Immunohistochemistry
Cardiac tissue was obtained from a 4-year-old boy with KD who died unexpectedly after complaining of chest pain on the 7th day of the disease. There was neither aneurysmal change in any coronary arteries nor ischemic change in any cardiac lesions. It was speculated that he had died from critical arrhythmia; he had not been treated with IVIG. The tissue was subjected to routine histological processing and was embedded in paraffin; thereafter, 6-μm-thick sections were subjected to immunoperoxidase staining with the Vectastain Elite ABC kit (Vector Lab, Burlingame, CA, USA), according to the manufacturer’s instructions. A monoclonal antibody specific for human MMP-9 was prepared as the primary antibody (Fujiyakuhin, Toyama, Japan). As a negative control, serial sections were incubated with mouse IgG (Vector Lab).

Detection of Plasma MMPs
MMP-1, -2, -9 and TIMP-1, -2 levels in plasma samples were measured using a 2-step sandwich ELISA assay system (Amersham Biosciences, Freiburg, Germany), according to the manufacturer’s instructions. The resultant color was measured at 450 nm in a microtiter plate spectrophotometer (Bio-Rad Laboratories, Richmond, CA, USA). The concentration of each MMP or TIMP was determined by interpolation from a standard curve. All assays were performed in duplicate.

Semi-Quantitative Reverse Transcription (RT)-Polymerase Chain Reaction (PCR)
Total RNA was isolated from non-serum-cultured HUVECs and fresh PBMCs using TRIZOL Regent (Invitrogen, San Diego, CA, USA). After the removal of residual DNA with DNase I (Nacalai Tesque, Kyoto, Japan), a total of 5 μg RNA was reverse transcribed in 20 μl using SuperScript II reverse transcriptase kit (Invitrogen). The primers of MMP-9 and GAPDH were prepared as described previously.15 PCR reactions were performed as follows: denaturing at 94°C for 30 s, annealing at 60°C for 30 s, and extension at 72°C for 45 s using the Taq polymerase kit Ex Taq (Takara, Tokyo, Japan). PCR products were separated by electrophoresis on a 2% agarose gel; the gel was stained with ethidium bromide and photographed using a CCD image system (Toyobo FASIII; Toyobo, Tokyo, Japan). The signal densities of the bands were calculated using a molecular analyzing software (Quantity One ver. 2.5; PDI Ltd, NY, USA). To ensure that the PCR amplification was indeed semi-quantitative, the numbers of cycles and concentrations of cDNA were varied in the linear phase of the exponential amplification curve. Further, for each newly synthesized cDNA, the abundance of HUVEC or PBMC cDNA was calculated, and then used as a reference point to adjust the amount of cDNA for each sample for PCR amplification. The signal ratio of MMP-9/GAPDH was calculated and expressed as the MMP-9 mRNA index.

Statistical Analysis
Unless indicated, results are expressed as mean±standard deviation (SD) or mean±standard error of the mean (SEM) of n observations. Data were analyzed by Student’s t-test or non-parametric U-test using StatView ver. J4.5 (Abacus Concepts, Calabasas, CA, USA). Statistical difference was considered as significant if P<0.05.

Table. Plasma Levels of MMPs and TIMPs

<table>
<thead>
<tr>
<th>Control</th>
<th>KD</th>
</tr>
</thead>
<tbody>
<tr>
<td>NF (n=15)</td>
<td>F (n=25)</td>
</tr>
<tr>
<td>MMP-1</td>
<td>35.1±2.3</td>
</tr>
<tr>
<td>MMP-2</td>
<td>389.3±169.8</td>
</tr>
<tr>
<td>TIMP-2</td>
<td>58.8±32.9</td>
</tr>
<tr>
<td>MMP-9</td>
<td>31.8±12.1</td>
</tr>
<tr>
<td>TIMP-1</td>
<td>167.8±40.0</td>
</tr>
</tbody>
</table>

Data were measured in duplicate by 2-step sandwich ELISA and expressed as the mean ± SD (ng/mL).

P<0.001 vs *NF, vs *F, vs *KD I.

The plasma levels of both MMP-9 and TIMP-1 were significantly higher in KD I than in groups H and F or in later KD phases (P<0.001). There were no significant differences in the plasma levels of MMP-9 or TIMP-1 between KD patients with and without coronary arterial lesions.

MMP, matrix metalloproteinase; TIMP, tissue inhibitor of MMP; KD, Kawasaki disease; NF, non-febrile controls; F, febrile controls; I, within 7 days of onset of disease before intravenous high-dose γ-globulin therapy (IVIG); II, immediately after IVIG; III, 1 month from the onset of the disease; ND, not done; H, healthy controls.
Informed Consent
In our all studies using clinical samples, we obtained consent from the parents or guardians of the subjects.

Results
Plasma MMP-1, -2, -9, and TIMP-1, -2
No significant differences in the expression of MMP-1 and -2 were detected among the non-febrile controls or among the KD patients in all phases (Table). The plasma levels of both MMP-9 and TIMP-1 markedly increased in the febrile controls (group F) and in the KD patients in all phases (I, II, III) as compared with the non-febrile controls (group NF). Plasma levels of MMP-9, TIMP-1, and MMP-9/TIMP-1 were significantly higher in KD phase I than in group F or in later phases of KD (Table).

MMP-9 Immunohistology
Diffuse and strong staining of MMP-9 was observed in the coronary lesion surrounding the anterior descending branch of the left coronary artery and whole cardiac muscle of the child who died from KD (Figures 1A, B).

Figure 1. Immunohistological staining for MMP-9 in a coronary lesion of a KD patient who died in the acute stage of the disease (7 days from onset). Staining shows MMP-9 in lesions around the main trunk of the left anterior descending branch (A×40, B×200, or negative control C×200). Strong staining for MMP-9 can be seen diffusely in the arterial lesions and myocardium of the KD patient (A, B). KD, Kawasaki disease; MMP, matrix metalloproteinase.

Figure 2. Expression of MMP-9 mRNA in HUVECs. The indices of MMP-9 mRNA in groups H, F, KD I, II, and III were 3.7±3.0, 5.5±2.6, 41.2±48.8, 38.1±39.5, and 30.6±43.1, respectively. Plasma samples obtained from patients in all phases of KD significantly stimulated the production of MMP-9 by HUVECs as compared with the healthy and febrile control groups. Data are expressed as mean±SEM. HUVECs, human umbilical endothelial cells. Other abbreviations are as in Table.
Expression of MMP-9 Transcripts in HUVECs or PBMNCs

Involvement of ECs and PBMNCs in the synthesis of MMP-9 during KD was determined by semi-quantitative RT-PCR analysis of MMP-9 mRNA in HUVECs and PBMNCs. Following non-serum incubation, HUVECs were treated overnight at 37°C with each plasma sample at a final concentration of 5%. Expression of MMP-9 mRNA was significantly elevated in HUVECs treated with the plasma from all phases of KD compared with both the healthy and febrile control groups (Figure 2). To evaluate the possible role of cytokines in the synthesis of MMP-9, HUVECs were treated with IFN-γ, TNF-α, IL-1β, and IL-6 at various concentrations, as well as with the plasma samples. The expression of MMP-9 mRNA in HUVECs was strongly enhanced by IL-1β, IL-6 or TNF-α, but suppressed by IFN-γ, in a dose-dependent manner (Figure 3). To confirm cytokine regulation of MMP-9 synthesis by ECs in KD, inhibition tests were performed using specific anti-human IFN-γ, IL-1β, IL-6, or TNF-α monoclonal antibodies against the plasma samples, as described previously. However, no inhibitory effect was observed (data not shown). Other than in a few patients, MMP-9 mRNA expression in PBMNCs was not significantly increased, even in KD phase I (group NF 4.01±4.47; KD I 7.11±9.04, II 5.30±5.86, III 2.90±4.70, mean±SD, respectively).

Expression of TIMP-1 Transcripts in HUVECs

The synthesis of TIMP-1 in ECs during KD was also examined by the same methods as for MMP-9, and expressed as the TIMP-1 mRNA index. There was no significant increase of the TIMP-1 mRNA index in either group NF or in any phases of KD (group NF 4.84±10.00; KD I 0.77±0.83, II 1.79±2.72, III 0.77±0.86, mean±SD, respectively).

Discussion

Our study showed that circulating MMP-9 levels are markedly increased in KD, while MMP-1, -2 and TIMP-2 levels showed no significant change as compared with the control groups. Previous reports have been that MMP-1, 2, 3 and TIMP-2 levels are significantly higher in the acute stage of KD than in controls. These differing results may be related to the type of ELISA (i.e., 1- or 2-step sandwich) or the anti-MMP monoclonal antibodies used in assay. Those reports also showed that the levels of both MMPs and TIMPs were increased in other febrile patients. Nevertheless, there was no significant difference in the ratio of MMP/TIMP between febrile patients and non febrile controls in the present study. Compared with other febrile diseases, the pathogenesis of KD is complex because of the various interactions among increased immunological substances, immunological cells, and vascular component cells. It is too difficult to explain the controversy regarding the imbalance of MMP/TIMP between KD and other febrile conditions. However, it is strongly suggested that the complicated pathogenesis of KD, including the imbalance of MMP/TIMP, could play an important role in the development of vascular remodeling in this particular disease. In our study, MMP-9 expression was detected in the entire myocardium of patients with acute KD, and staining revealed the presence of MMP-9 within the EC. These results suggest that most individual monocytes, neutrophils or myocardial cells express MMP-9. Our study showed that HUVECs, but not PBMNCs, are a source of MMP-9 in KD. ECs secrete various MMPs, including MMP-9, thereby facilitating the degradation of the basement membrane, a process that is essential for cell migration. Migration of ECs is stimulated in the pre-IVIG phase of KD, offering support for the hypothesis that activated ECs are a major producer of MMP-9 in KD. Semi-quantitative RT-PCR showed that MMP-9 expression in an EC line was stimulated by treatment with plasma from patients in the pre-IVIG phase of KD. MMP-9 expression can be upregulated by several factors activated in KD. IFN-γ, IL-1β, IL-6, and TNF-α are the major cytokines that are increased in the acute phase of KD, and thus in the present study, their effect on MMP-9 expression in ECs was examined. IL-1β, IL-6, and TNF-α were all stimulatory, whereas IFN-γ decreased the expression of MMP-9. This finding suggests that cytokines in the plasma of patients act as important regulators of MMP-9 synthesis in ECs. To confirm this possibility, the effect of inhibiting the cytokines was examined by treating the plasma samples with neutralizing anticytokine antibodies, but they did not significantly inhibit MMP-9 expression in ECs. This unexpected result might be explained by a combined effect of the cytokines, because various cytokines in plasma simultaneously regulate the expression of MMP-9. Therefore, it may be difficult to demonstrate a neutralizing effect for any single anti-
cytokine antibody. Furthermore, because MMP-9 expression was more increased in the later phases of KD, the presence of other regulators, for example, super-antigen, superoxide etc, apart from cytokines in plasma samples should be investigated.

MMP-9 expression is also regulated by enzyme inhibitors. The endogenous inhibitors of MMP-9 are the TIMPs, with TIMP-1 and TIMP-2 having preferred selectivity for inhibiting MMP-9 and MMP-2, respectively. For diseases in which excessive matrix degradation occurs, the balance between MMPs and TIMPs is often upset, resulting in an overall increase in MMP activity. It is well known that TIMP-1 is constitutively secreted by various cells including ECs. Thus, ECs produce both MMPs and TIMPs, regulating degradation and reconstruction of the vascular basement membrane. In the present study, we confirmed that plasma levels of TIMP-1 are increased throughout the acute to subacute phases of KD, but decreased by IVIG therapy. It is possible that the increase in the level of TIMP-1 is a reaction secondary to the increase in MMPs. Therefore, the ratio of MMP-9/TIMP-1 might be a better index, reflecting the activity of MMP-9 in arterial lesions, rather than the level of MMP-9 alone. Notwithstanding the normalization of plasma levels on inflammatory reactions or inflammatory cytokines, the ratio of MMP-9/TIMP-1 showed a high value even 1 month after the onset of KD. Before IVIG therapy, the active inflammation of coronary arterial lesions (CAL) gradually improved after day 25 of KD and almost disappeared by day 40. From our study results, we could not suggest that ECs are a major source of TIMP-1, which may be the limitation of the in vitro assay. Therefore we need to study other vascular component cell-lines, including smooth muscle cells, fibroblasts, and myocardial cells. However, excessive serum TIMP-1 causes accumulation of collagen fibers synthesized by fibroblasts, and in KD, intimal hypertrophy of CAL could result in ischemic heart disease. Therefore, it is probable that the balance of MMPs and TIMPs is important in vascular remodeling, during the destruction of vascular walls and in repair or intimal hypertrophy. In addition, the relationship between blood vessel components, MMPs, and TIMPs in the local lesions of vasculitis needs to be evaluated in relation to the time-dependent and histological changes observed, in order to gain improved understanding of the influence of MMPs and TIMPs in KD vasculitis. Clinical evaluation of this system poses many challenges. We conclude that in vivo animal models could be useful for evaluating the serial expression of MMPs and TIMPs by ECs in the local lesions of KD vasculitis, in order to promote further understanding of the etiology of this most common acquired pediatric cardiac disease.

References


