Elevated Levels of Matrix Metalloproteinase 9 and Tissue Inhibitor of Metalloproteinase 1 during the Acute Phase of Kawasaki Disease

Pong Kian Chua, Marian E. Melish, Qigui Yu, Richard Yanagihara, Kara S. Yamamoto, and Vivek R. Nerurkar

Retrovirology Research Laboratory, Pacific Biomedical Research Center, and Department of Pediatrics, John A. Burns School of Medicine, University of Hawaii at Manoa, Honolulu, Hawaii 96822

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Kawasaki disease (KD) is an acute, self-limiting, multisystem vasculitis of unknown etiology affecting infants and young children. Unless treated promptly with high-dose intravenous gamma globulin and aspirin, patients frequently develop coronary aneurysms. Previously, matrix metalloproteinase 9 (MMP-9), which is secreted complexed to tissue inhibitor of metalloproteinase 1 (TIMP-1), has been implicated in abdominal aortic aneurysm formation. Since the clinical and pathological features of KD include inflammation and weakening of blood vessels, we analyzed acute- and convalescent-phase paired plasma or serum samples from 31 KD patients, 7 patients who did not completely meet the criteria for KD, and 26 non-KD controls (9 febrile and 17 afebrile patients) for pro-MMP-9 (92 kDa) enzyme activity by gelatin zymography and for active MMP-9 (83 kDa), pro-MMP-9, and TIMP-1 protein levels by enzyme-linked immunosorbent assay. Statistical analysis was performed by using Student t tests, linear regression, and the Wilcoxon rank-sum test. Markedly elevated pro-MMP-9 enzymatic activity, pro-MMP-9 protein levels, and TIMP-1 protein levels were found during the acute phase of illness in patients with clinically established KD and in patients who were suspected of having KD but did not meet all of the criteria. There was no significant difference in active MMP-9 levels. Furthermore, pro-MMP-9 and TIMP-1 protein levels were significantly elevated among KD patients, compared to those of febrile and afebrile non-KD controls. The significantly elevated pro-MMP-9 enzyme and protein levels during the acute phase of KD may reflect vascular remodeling or an inflammatory response to a microbial agent, suggesting a pathophysiological role for MMP-9 in coronary aneurysm formation.

MATERIALS AND METHODS

Study population. Patients who presented with KD or with other acute febrile or afebrile illnesses at the Kapiolani Medical Center for Women and Children (KMCWC) in Honolulu, Hawaii, from 1996 to 2000 were studied after informed consent was obtained from their parents or guardians. Blood specimens were processed within 24 h of collection. Plasma samples from 35 KD patients and serum samples from 3 KD patients were stored at −30°C until further use. Plasma from all controls was separated and stored as described above. Blood samples from 38 KD patients were drawn during the acute phase of illness (before administration of IVIG) and during convalescence (6 weeks to 3 months after disease onset), when patients no longer exhibited clinical features of KD and elevated inflammatory markers of C-reactive protein and the erythrocyte sedimentation rate had returned to normal. This study was approved by the KMCWC institutional research and ethics committee.

Gelatin zymography. Protein concentrations in plasma or serum samples from KD and non-KD patients were measured with the Bio-Rad D1 Protein Assay Kit (Bio-Rad, Hercules, Calif.). Pro-MMP-2 and pro-MMP-9 enzyme activities were measured by gelatin zymography as previously described (26). Briefly, 15 µg of...
total protein was mixed with electrophoresis sample buffer (20% [wt/vol] glyc erol, 4% [wt/vol] sodium dodecyl sulfate [SDS], 62.5 mM Tris-HCl [pH 6.8], 0.1% [wt/vol] bromophenol blue) and loaded onto an 7.5% SDS-polyacrylamide gel containing 1 mg of type B gelatin from bovine skin (Sigma Chemical Co., St. Louis, Mo.) per ml. Gel electrophoresis was performed at 120 V for 1.5 h, and the gel was washed six times for 5 min each time with wash buffer containing 50 mM Tris-HCl (pH 7.4), 5 mM CaCl₂, 0.01% [wt/vol] Na₂MoO₄, and 2.5% [vol/vol] Triton X-100 to remove the SDS from the gel and rinsed twice for 5 min each time in incubation buffer (same as wash buffer but without Triton X-100) and incubated overnight (18 h) in the incubation buffer. Gels were stained with Coomassie brilliant blue (0.1% [wt/vol] G250 in 50:20:30 methanol-acetic acid-distilled water) for 30 min and destained in destaining buffer (30:1:69 methanol-acetic acid-distilled water). Clear bands against a blue background, indicating gelatin lysis, were observed at 72 and 92 kDa, which are equivalent to pro-MMP-2 and pro-MMP-9 enzyme activities, respectively (Fig. 1). To quantitate the amount of enzymatic activity, we used the AppCollage PPC4.0 Image Analysis software program (Fotodyne Inc., Hartland, Wis.). The amount of gelatin lysis was captured, and the band intensity was calculated on the basis of pixel counts and normalized against the background.

ELISA. Pro-MMP-9 total protein, active MMP-9 enzyme, and TIMP-1 protein levels were measured with sandwich enzyme-linked immunosorbent assay (ELISA) kits (RPN 2614, RPN 2634, and RPN 2611, respectively; Amersham Pharmacia Biotech Company, Piscataway, N.J.) used in accordance with the manufacturer’s instructions. The pro-MMP-9 kit measures both human pro-MMP-9 and pro-MMP-9 complexed to TIMP-1 but not active MMP-9, while the TIMP-1 kit measures both human free TIMP-1 protein and TIMP-1 that is complexed with MMP-1, MMP-2, MMP-3, and MMP-9. For our purposes, the MMP-9 activity ELISA kit was used to measure endogenous levels of active MMP-9. To determine enzyme and protein stability, five random samples from various collection dates were frozen and thawed twice and tested by ELISA for MMP-9. To determine enzyme and protein stability, TIMP-1 kit measures both human free TIMP-1 protein and TIMP-1 that is complexed with MMP-1, MMP-2, MMP-3, and MMP-9. For our purposes, the TIMP-1 activity ELISA kit was used to measure endogenous levels of active TIMP-1. To determine enzyme and protein stability, five random samples from various collection dates were frozen and thawed twice and tested by ELISA for TIMP-1.

Statistical analysis. Student t tests, linear regression, and the Wilcoxon rank-sum test were employed to calculate the P values and coefficients of correlation of pro-MMP-9 and TIMP-1 protein concentrations, pro-MMP-9 protein and enzyme levels, and pro-MMP-9 enzyme levels between the patients and controls.

RESULTS

Study population. KD patients were classified as having complete or incomplete KD on the basis of their complete or incomplete fulfillment of the diagnostic clinical criteria established by the Kawasaki Study Group (41). All 38 KD patients were judged by one of two experienced clinicians (M.E.M. or K.S.Y.). Diagnoses of febrile patients were fever without focus, negative blood culture (n = 4), gastroenteritis (n = 2), pneumococcal bacteremia (n = 1), sinusitis (n = 1), and encephalomyelitis (n = 1), while afebrile patients were diagnosed with gastroenteritis and dehydration. Plasma or serum samples were available from patients classified as presenting with complete or incomplete KD and febrile and afebrile non-KD patients (Table 1). Of the 38 KD patients, 8 were Japanese, 2 were Korean, 1 was Japanese-Korean, 2 were Micronesian, and the remaining 25 were Filipino (n = 8), Chinese (n = 7), part Hawaiian (n = 2), Caucasian (n = 1), Vietnamese (n = 1), and an admixture (n = 6) of one or more of the aforementioned ethnicities.

TABLE 1. Study population

| Group       | No. of: | Total | Median age (mo) (range) |
|-------------|---------|-------|------------------------|
| KD          |         |       |                        |
| Complete    | 17      | 31    | 17.5 (1–96)            |
| Incomplete  | 5       | 7     | 19 (4–48)              |
| Non-KD      |         |       |                        |
| Febrile     | 3       | 9     | 36 (6–72)              |
| Afebrile    | 7       | 17    | 35 (8–84)              |

MMP-2 and MMP-9 enzyme activity. A representative gelatin zymogram, with gelatin lysis at 92 and 72 kDa, corresponding to the pro-MMP-9 and pro-MMP-2 enzymes, respectively, is shown in Fig. 1. The gelatinase activity was inhibited with 10 mM EDTA (data not shown), thus verifying that the gelatin lysis was due to the pro-MMP-9 and pro-MMP-2 enzymes. Elevations in pro-MMP-9 enzyme activity were observed in acute-phase plasma samples from patients with complete and incomplete KD, compared to the respective convalescent-phase samples (Fig. 2A and Table 2). Patients with complete KD had significantly elevated levels of pro-MMP-9 compared to febrile and afebrile non-KD patients, whereas there was no statistically significant difference in pro-MMP-9 enzyme activity between incomplete-KD patients and febrile and afebrile non-KD patients. Additionally, there was no statistically significant difference in pro-MMP-9 enzyme levels between complete- and incomplete-KD patients in the acute phase (Table 2). Comparisons of various groups by the nonparametric test concurred with results obtained with the Student t test for MMP-9 enzyme activity and the Wilcoxon rank-sum test for TIMP-1 protein concentrations. Pro-MMP-2 enzyme levels in the acute phase of complete-KD patients were not significantly higher than those of the convalescent-phase and afebrile patients but were significantly elevated compared to those of febrile patients (Table 2). For incomplete-KD patients, the acute-phase pro-MMP-2 levels were not significantly elevated compared to convalescent-phase, febrile, and afebrile patients. There was also no significant difference between complete- and incomplete-KD patients in the acute phase (Table 2).

MMP-9 and TIMP-1 protein concentrations. Mean pro-MMP-9 protein concentrations were significantly elevated in the acute-phase samples of KD patients, compared to the respective convalescent-phase samples, as well as compared to samples from febrile and afebrile non-KD patients (Fig. 2B and Table 2). Similarly, mean TIMP-1 protein concentrations were significantly elevated in the acute phase of KD compared to the respective convalescent phase, as well as compared to those of febrile and afebrile non-KD patients (Fig. 2C and Table 2). As observed previously, transient dilated coronary aneurysm (TDCA) or coronary artery aneurysm (CAA) pa-
FIG. 2. (A) Scatter plot showing significantly elevated pro-MMP-9 enzyme activity in acute-phase specimens from patients with complete and incomplete KD, compared to the respective convalescent-phase samples ($P < 0.01$ and $P < 0.05$, respectively). There was a significant increase in the pro-MMP-9 enzyme levels in the acute phase of complete-KD patients compared to those of febrile ($P < 0.01$) and afebrile ($P < 0.01$)
patients did not have concentrations of pro-MMP-9 or TIMP-1 protein in their plasma greater than the mean pro-MMP-9 or TIMP-1 protein levels in the acute phase of complete KD, respectively.

Active MMP-9 levels measured with an ELISA kit showed no statistically significant difference between acute-phase KD patient plasma and the respective convalescent-phase plasma, KD patients and febrile controls, and complete-KD patients and afebrile controls (Table 2). We did, however, observe a statistically significant difference between incomplete-KD patients and afebrile controls, which could have been an artifact due to the small sample size in the incomplete-KD group. These results indicate that although pro-MMP-9 levels are higher in the plasma or serum of KD patients, not all pro-MMP-9 is activated into the active form of MMP-9 (83 kDa).

With the Student t test and the Wilcoxon rank-sum test, no statistically significant difference was found in pro-MMP-9 protein (P = 0.15) or enzyme (P = 0.26) activities or in TIMP-1 protein levels (P = 0.37) between acute-phase patients who presented at KMCWC before day 5 (n = 23) or between days 6 and 9 (n = 8) after the onset of clinical symptoms. Linear regression analysis showed a significant correlation between pro-MMP-9 protein levels and pro-MMP-9 enzyme activity during the acute phase of complete (r = 0.44, P < 0.01) and incomplete (r = 0.81, P < 0.01) KD, whereas no such correlation was observed in febrile non-KD patients (r = 0.17, P = 0.26) or in afebrile non-KD patients (r = 0.05, P = 0.36). A positive correlation was also found between pro-MMP-9 and TIMP-1 proteins levels in the acute phase of complete KD (r = 0.18, P < 0.02) but not in patients with incomplete KD (r = 0.22, P = 0.29) or among febrile (r = 0.02, P = 0.64) or afebrile non-KD patients (r = 0.11, P = 0.19). There was no statistically significant difference in pro-MMP-2 and pro-MMP-9 enzyme activity, pro-MMP-9 and TIMP-1 protein levels, and active MMP-9 levels after freezing and thawing of five random samples collected on various dates. Similarly, exclusion of three serum samples from the analysis did not lead to any difference in the data presented.

**DISCUSSION**

MMP-9 plays a major role in many physiological activities and pathological conditions. Failure to regulate these functions can be detrimental. For example, pro-MMP-9, as well as active MMP-9, levels are significantly elevated among patients with abdominal aortic aneurysms (5, 12, 18, 23, 24). Since the clinical presentation of KD involves vasculitis and coronary aneurysm, we posited that MMP-9 enzyme and protein levels are elevated in KD patients. Recently, three Japanese groups have independently reported elevated levels of MMP-9, TIMP-1, and neutrophil elastase in the serum or plasma of KD patients prior to infusion of IVIG (32, 35; K. Sakata, Z. Onouchi, K. Hamaoka, and Y. Yamamoto, Abstr. 6th Int. Kawasaki Dis. Symp., abstr. 24, p. 21). Our study is the first outside of Japan to demonstrate markedly elevated pro-MMP-9 enzyme and protein levels during the acute phase of KD. The uniformity of this response among Japanese patients, as well as among non-Japanese patients of various ethnic backgrounds in Hawaii, suggests that MMP-9 may play a central role in the pathophysiology of KD.

Cellular sources of pro-MMP-9 include smooth-muscle cells and B and T lymphocytes (14, 21, 24, 37). More recently, Takeshita and colleagues demonstrated that the level of MMP-9 in serum was increased in KD patients and that this elevation correlated with the number of circulating leukocytes (35). We have observed that peripheral blood mononuclear cells obtained during the acute phase of KD, when cultivated in serum-free medium, secrete high levels of pro-MMP-9 enzyme (Q. Yu, P. K. Chua, and V. Nerurkar, unpublished data). Collectively, these data suggest that immune cells are the main source of pro-MMP-9 in KD.

By using gelatin zymography, we were able to detect pro-MMP-9 (92 kDa) enzyme activity and negligible amounts of active MMP-9 (83 kDa) in all of our KD patients and controls. We performed ELISAs to detect endogenous levels of active MMP-9. The commercially available MMP-9 activity ELISA kit cross-reacts with both pro-MMP-9 and active MMP-9 and includes a step that activates all pro-MMP-9 into active MMP-9. Since we were only interested in measuring endogenous levels of active MMP-9, we did not activate pro-MMP-9. We detected very low levels of endogenously active MMP-9 by ELISA, with no statistically significant difference between KD (complete and incomplete) patients and febrile controls or between complete-KD patients and afebrile controls, but did observe a statistically significant difference between incomplete-KD patients and afebrile controls. In contrast, Takeshita et al. measured levels of active MMP-9 by first activating pro-MMP-9 in serum with 4-aminophenylmercuric acetate into active MMP-9 (35). They demonstrated that the level of active MMP-9 was higher in the acute phase of KD than in the convalescent phase and among healthy children. These data differ from our experimental setup, in which we specifically assayed for pro-MMP-9 and active MMP-9 levels separately. Our results showed that pro-MMP-9 levels are elevated in the acute phase of KD compared to those in convalescent-phase, febrile, and afebrile patients, whereas active levels of MMP-9 were not different between the groups. Our data are more indicative of the levels of proMMP-9 and active MMP-9 in the circulatory system.

Collectively, these results indicate that pro-MMP-9 levels are higher in KD patients and that very little pro-MMP-9 is activated in the plasma. The extremely small amount of active non-KD patients. No statistically significant difference was found between incomplete-KD patients and febrile (P = 0.13) and afebrile (P = 0.1) non-KD patients. (B) Scatter plot revealing markedly elevated pro-MMP-9 protein levels in the acute phase of complete and incomplete KD compared to those of the respective convalescent-phase (both P < 0.01), febrile (P < 0.01 for complete, P < 0.05 for incomplete), and afebrile (both P < 0.01) patients. (C) TIMP-1 protein levels were elevated in acute-phase specimens of patients with complete and incomplete KD, compared to those in the respective convalescent-phase samples (both P < 0.01) and febrile (both P < 0.01) and afebrile (both P < 0.01) patient samples. Mean and median protein levels for all study populations are indicated by lines and arrows, respectively. Filled circles denote TDCA or CAA patients. Statistical significance at the 1 and 5% levels is indicated by one and two asterisks, respectively. Conv, convalescent.
TABLE 2. MMP-9, TIMP-1, and MMP-2 enzyme and protein levels

| Diagnosis (no. of patients) | MMP-9 | TIMP-1 | MMP-2 |
|----------------------------|-------|--------|-------|
|                            | Enzyme (pixel count) | Protein (ng/ml) | Activity (ng/ml) | Enzyme (pixel count) | Protein (ng/ml) | Activity (ng/ml) |
| Complete KD (31)           |       |        |       |       |        |        |
| Acute phase                | 114,754<sup>a</sup><sup>b</sup><sup>c</sup> | 83,843 | 12,630–29,511 | 1,114<sup>b</sup> | 645 | 30–4,206 | 11 | 7 | 1–122 | 1,321<sup>b</sup> | 1,218 | 152–2,515 |
| Convalescent phase         | 25,523 | 16,927 | 2,250–113,540 | 193 | 129 | 26–1,013 | 9 | 9 | 1.2–20.7 | 651 | 400 | 120–2,500 |
| Incomplete KD (7)          |       |        |       |       |        |        |
| Acute phase                | 95,222<sup>a</sup><sup>b</sup><sup>c</sup> | 76,512 | 22,047–253,990 | 1,271<sup>b</sup> | 1,193 | 252–2,630 | 8.4<sup>b</sup> | 8 | 2.5–15.2 | 1,487<sup>b</sup> | 1,534 | 585–2,493 |
| Convalescent phase         | 21,118 | 9,118 | 4,017–58,511 | 109 | 84 | 31–322 | 16 | 15 | 7.4–31.1 | 412 | 330 | 222–926 |
| Febrile (9)                | 54,948 | 29,693 | 1,507–190,606 | 428 | 141 | 62–2,005 | 9 | 8 | 2.3–16.7 | 599 | 228 | 55–2,498 |
| Afebrile (17)              | 25,071 | 15,258 | 3,062–268,922 | 110 | 55 | 10–808 | 16 | 16 | 1.5–33.8 | 552 | 439 | 106–2,824 |

<sup>a</sup>P < 0.05, acute versus convalescent phase.<br><sup>b</sup>P < 0.05, acute phase versus febrile.<br><sup>c</sup>P < 0.05, acute phase versus afebrile.
febrile patients. This difference could possibly be due to inhibition of pro-MMP-2 by TIMP-2 in febrile patients. Also, Senzaki et al. (32) reported that the MMP-2 level is elevated in the convalescent phase after IVIG administration and also higher in KD patients than in afebrile patients. The difference between their results and ours could be due to the difference between the assays used, in that the sandwich ELISA used by Senzaki et al. (32) could have detected both MMP-2 and MMP-2 complexed with TIMP-2, whereas zymography would have just picked up pro-MMP-2.

Our study population of incomplete-KD patients was small, and this could help explain the lack of correlation between pro-MMP-9 and TIMP-1 protein levels in the acute phase and perhaps explain the statistically significant difference in active MMP-9 levels between incomplete-KD patients and afebrile controls. However, the small sample size does not discount our findings that incomplete-KD patients have elevated levels of both pro-MMP-9 enzyme and pro-MMP-9 protein, as well as TIMP-1 protein, during the acute phase of illness. Compared to febrile and afebrile non-KD patients, patients with incomplete KD still had higher pro-MMP-9 and TIMP-1 protein levels. Overall, we measured MMP-9 protein and enzyme levels by ELISA and zymography, respectively. Takeshita et al. measured MMP-9 mRNA levels by RT-PCR and MMP-9 levels by ELISA, and Senzaki et al. and Sekiguchi et al. measured MMP-9 levels by ELISA (31, 32, 35). All three groups independently demonstrated elevated levels of MMP-9 in the acute phase of KD compared to the convalescent phase or controls. This, coupled with the significant correlation between pro-MMP-9 protein levels and pro-MMP-9 enzyme activity during the acute phase of complete and incomplete KD, strongly suggests that MMP-9 plays an important role in the pathogenesis of KD and is an inflammatory marker for KD.

Polymorphism in the MMP-9 gene promoter has also been reported among intracranial aneurysm and myocardial infarction patients (25, 44). Furthermore, polymorphisms in the MMP-3 promoter and the gene for TIMP-1, but not the gene for MMP-9, have been reported to be associated with abdominal aortic aneurysm (39, 42). A few patients in our KD study population had extremely high pro-MMP-9 enzyme or pro-MMP-9 protein levels. It is tempting to speculate that these patients may have a specific polymorphism in the gene for MMP-9 that resulted in overproduction of pro-MMP-9. Alternatively, these patients may have elevated levels of other cytokines that could have elicited overproduction of pro-MMP-9. We also noticed that KD patients who had extremely high levels of pro-MMP-9 did not necessarily experience CAA or TDCA. This implies that other factors and/or molecules other than MMP-9 may be synergistically involved in causing CAA or TDCA.

The histopathology of KD shows increased monocytes, macrophages, and immunoglobulin A-secreting plasma cells at sites of vascular lesions (28), with ruptured internal elastic lamina, intimal hypertrophy, and degeneration of the basement membrane. More recently, Gavin et al., using immunohistochemical analysis of MMP-2 and MMP-9 in paraffin-embedded formalin-fixed coronary artery tissue from 11 fatal acute KD cases, demonstrated significant differences in the expression of MMP-9 in KD CAA (7). Moreover, they demonstrated high expression of MMP-9 in mononuclear inflammatory cells in CAA compared to that in nonaneurysm KD coronary arteries or in control arteries (7). Available data, therefore, strongly suggest a pathophysiological process by which MMP-9 may cause coronary aneurysms in KD patients. Furthermore, since TIMP-1 is the natural inhibitor of MMP-9, it is tempting to suggest the possibility of using TIMP-1 as a therapeutic approach in treating KD patients to prevent coronary aneurysms.

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