·Original Article in English·

Matrix metalloproteinase29 expression in the peripheral blood of children with Kawasaki disease and its relationship with coronary artery lesions

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Abstract: Objective Matrix metalloproteinase29 (MMP29), a metalloproteinase, is capable of degrading type IV, V collagens, as well as gelatins. Increased levels of MMP29 have been detected in aortic aneurysms in adult human, suggesting its role in arterial wall destruction and aneurysm formation. This study was designed to investigate the potential role of MMP29 in the pathogenesis of coronary artery lesions in Kawasaki disease (KD) patients. **Methods** Twenty2seven children with KD [17 with coronary artery lesions (CALs) and 10 without] and age2matched 10 febrile and 10 healthy controls were enrolled in this study. Gelatin zymography and ELISA were used to detect the activity and levels of serum MMP29. MMP29 mRNA expression in the leucocytes was detected using reverse transcription2polymerase chain reaction (RT2PCR). **Results** 1) The activity (10.2 ±2.2) and levels of MMP29 (1 116 ±691 ng/ml) in KD patients with CALs in the acute phase were significantly higher than those without CALs (6.2 ±2.1, 457 ±133 ng/ml, respectively; $P < 0.05$). Both of them in either the KD patients with CALs or without were higher than those of the healthy controls (0.1 ±0.0, 72 ±24 ng/ml, respectively; $P < 0.01$) and the febrile controls (3.1 ±1.4, 221 ±154 ng/ml, respectively; $P < 0.05$). 2) There was a significantly positive correlation between the serum MMP29 protein levels and the circulating leucocytes counts in KD patients in the acute phase ($r = 0.480$, $P < 0.05$). 3) The MMP29 mRNA expression in the leucocytes of KD patients in the acute phase were significantly elevated, as compared with the febrile and healthy controls ($P < 0.01$). There were no significant differences in the MMP29 mRNA expressions between the two KD groups. 4) The activity, protein levels and mRNA expression of MMP29 in the KD patients decreased obviously from the subacute through the convalescent phases, as compared with the acute phase ($P < 0.01$). **Conclusions** The MMP29 expression in KD patients in the acute phase was significantly elevated, especially in those with CALs. MMP29 may be involved in the development of coronary artery lesions in KD. **[Chin J Contemp Pediatr, 2004, 6(6): 456 - 461]**

Key words: Kawasaki disease; Coronary artery lesions; Matrix metalloproteinase29

川崎病患儿外周血基质金属蛋白酶29 的表达及其与冠状动脉损伤的关系

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[**摘 要**] **目的** 观察 MMP29 在川崎病(KD)患儿不同时期表达水平,探讨其在冠状动脉损伤中的可能作用。**方法** 选择 KD 患儿 27 例为研究对象(无冠脉损伤组 10 例,冠脉损伤组 17 例),取年龄相仿的 10 例败血症患儿、10 例健康儿童为发热对照组和正常对照组。分别应用明胶酶谱法、酶联免疫吸附法检测血清 MMP29 的活性和蛋白浓度,半定量 RT2PCR 的方法检测外周血白细胞 MMP29mRNA 表达水平。**结果** (1)冠脉损伤组急性期血清 MMP29 活性和蛋白水平(10.2 ±2.2,1 146 ±691 ng/ml)较无冠脉损伤组显著增高(6.2 ±2.1,457 ±133 ng/ml, $P < 0.05$)。两组均较正常对照组(0.1 ±0.0,72 ±24 ng/ml)和发热对照组(3.1 ±1.4,221 ±154 ng/ml)显著增高($P < 0.01$或 $P < 0.05$)。(2) KD 患儿急性期血清 MMP29 蛋白水平与外周血白细胞计数显著正相关($r = 0.480$, $P < 0.05$)。(3)两组 KD 患儿急性期外周血白细胞 MMP29 mRNA 表达水平无显著性差异,但均显著高于发热对

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照组和正常对照组($P < 0.01$) 。(4)川崎病患儿急性期血清 MMP29 活性、蛋白浓度和白细胞表达 MMP29 mRNA 水平,在亚急性期、恢复期均依次明显降低($P < 0.01$) 。**结论** MMP29 在川崎病患儿急性期,尤其在伴冠状动脉损伤时表达明显升高;MMP29 可能参与了川崎病冠状动脉损伤的病理过程。

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[关 键 词] 川崎病;冠状动脉病变;基质金属蛋白酶29

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Kawasaki disease (KD) is an acute, self2limiting systemic vasculitis syndrome of unknown origin that mainly affects small and medium2sized arteries, particularly the coronary artery. Even after receiving intravenous immunoglobulin (IVIG) therapy, 3 % to 8 % of the KD children had subsequent coronary artery lesions (CALs). Ultimately approximately 4 % developed adult ischemic heart disease with myocardial infarction[1]. KD has become the most common cause of acquired heart disease in children. Matrix metalloproteinase29 (MMP29), which has a unique elastinolytic activity, can degrade most components of extracellular matrix and basement membrane protein, such as collagen and elastin. MMP29 is thought to play an important role in the pathogenesis of inflammatory vascular disease such as atherosclerosis[2] and abdominal aortic aneurysm[3] by degrading the internal elastic lamina. The aim of this study was to explore the potential role of MMP29 in the pathogenesis of coronary artery lesions in KD.

Materials and methods

Patients and samples preparation

Twenty2seven KD patients who had run a fever within 7 days and did not receive the IVIG and aspirin treatments before hospitalization between May 2003 and December 2003 were eligible for the study. All KD patients met the diagnostic criteria established by the Japanese KD Research Committee. CALs were defined when the diameter of the coronary artery was greater than 2.5 mm in patients aged $\leq$24 months or that was greater than 3.0 mm in patients aged > 24 months. Coronary aneurysm was not found in any of KD patients. The time of KD onset was defined as the day on which fever appeared. The disorder experienced 3 phases: acute (before the IVIG and aspirin therapy, on days 3 - 7 of the disease course), subacute (48 hours after IVIG therapy, on days 9 - 13) and convalescent phases (C2reactived protein recovery, on days 14 - 30). Once admitted, the KD patients were treated with IVIG (2 g/kg) infusion and oral aspirin (30 mg/kg per day). Ten children with fever (temperature > 38.5 ℃) and definitely diagnosed with septicemia were selected as febrile controls. Only antibiotics and acetaminophen had been administered for them before hospitalization. Ten aged2matched healthy children were used as normal controls. Blood samples were obtained from KD patients at 3 different phases, and from the 2 control groups. Serum samples were stored at - 80 ℃ for later use and the peripheral blood leucocytes (monocytes and neutrophils) were immediately isolated by density gradient centrifugation using Polymorphprep TM (Axis2Shield, Norway). Parental consent was obtained for each child enrolled in this study.

Population characteristics are shown in Table 1.

Gelatin zymography

MMP29 enzyme activity was detected using gelatin zymography as previously described[4]. Briefly, samples were prepared in non2reducing sample buffer (0.625 mM Tris2Hcl, 10 % glycerol, 2 % SDS, 2 % bromphenol blue), and electrophoresed on 10 % SDS2PAGE gels containing 1mg/ml gelatin (Sigma, USA). Gels were washed with 2.5 % Triton X2100 solution twice, for 30 minutes each time, and were incubated for 18 hours in a solution containing 50 mM Tris2HCl (pH 7.5), 10 mM $CaCl_2$, 200 mM NaCl, and 1 μM $ZnCl_2$. Then the gels were stained with 2.5 % (w/v) Coomassie brilliant blue G2250 in 50 ∶20 ∶30 methanol2acetic acid2distilled water for 1 hours, followed by being washed with 30 % (v/v) methanol and 1 % (v/v) acetic acid. Proteolytic bands were detected by examining unstained regions on the gelatin2stained background. Zymograms were subjected to densitometric analysis using Image J1.30

software. The relative MMP29 activity expression was calculated by multiplying the band area and the differences between the band intensity and the back2 ground intensity together.

ELISA

The total protein levels of MMP29 (proMMP29 and MMP29) in the sera were measured with an ELISA kit for human MMP29 (RD Company, USA). All procedures were performed strictly ac2 cording to the manufacturer's instructions.

Detecting the MMP29 mRNA expression using RT2 PCR

The total RNA was extracted from the isolated leucocytes (about 10^5 cells) by means of TRIzol (In2 vitrogen, USA). RNA concentration and purity were determined by absorbency at 260 and 280 nm. One μg RNA was used to synthesize cDNA in the presence of an oligodt152primer, RNase inhibitor and the M2MLV reverse transcriptase (Promega, USA) in a final volume of 40 μl. Sequence2specific oligonu2 cleotide primers were designed according to human MMP29 (sense primer: 5'2CCT TCT ACG GCC ACT ACT GT23', anti2sense primer: 5'2CCA CCT GGT TCA ACT CAC TC23'), or β2actin (sense primer: 5'2CAC GAT GGA GGG GCC GGA CTC ATC23', anti2sense primer: 5'2TAA AGA CCT CTA TGC CAA CAC AGT23'). PCR was performed in a 20 μl reaction system which contained 2 μl cDNA, 1 ×PCR buffer, 1.5 mM $MgCl_2$, 0.1 mM dNTP, 10 pM of each primer, 0.25 μl Taq DNA polymerase (Takara, Dalian). Amplification cycles were 95 ℃ for 5 minutes, followed by 30 cycles at 94 ℃ for 30 seconds, 61 ℃ for 30 sec2 onds, and 72 ℃ for 30 seconds and terminated by a final extension of 72 ℃ for 5 minutes. The PCR products for MMP29 and β2actin were 574 bp and 240 bp respectively, and were electrophoresed with 2 % agarose gel. The band intensity was determined by gel image analytic system (Gene Company, USA). MMP29 mRNA levels were ex2 pressed as intensity ratios to β2actin.

Statistical analysis

SPSS 10.0 for Windows was used and all data were presented as ($\bar{x} \pm s$). Differences were ana2 lyzed with the ANOVA test. Correlations were evalu2 ated with the Pearson correlation test. A P of less than 0.05 was considered significant.

Results

Laboratory findings

As shown in Table 1, the mean counts of white blood cells (WBC), neutrophils and monocytes were significantly higher in KD patients and febrile controls than normal controls ($P < 0.01$). A significant dif2 ference was also observed in the mean counts of WBC and neutrophils between the KD patients with CALs and without ($P < 0.05$).

Table 1 Population characteristics ($\bar{x} \pm s$)

Group	n (male/female)	Age (month)	Body weight (kg)	WBC ($\times 10^9$/L)	Neutrophils ($\times 10^9$/L)	Monocytes ($\times 10^9$/L)
Normal controls	10(5/5)	15.9 ±4.8	13.6 ±1.7	6.1 ±1.1	3.6 ±0.7	0.9 ±0.4
Febrile controls	10(5/5)	25.9 ±8.6	14.9 ±2.0	15.0 ±0.7[a,c]	9.2 ±4.3[a,c]	1.5 ±0.6[a]
KD						
Non2CALs	13(7/6)	16.4 ±8.5	11.0 ±2.1	13.4 ±5.8[a]	8.5 ±4.9[a]	1.6 ±1.5[a]
CALs	17(10/7)	23.0 ±15.6	12.4 ±2.8	20.7 ±8.1[b,c]	14.7 ±7.5[b,c]	1.5 ±0.9[a]

Note: a vs Normal controls $P < 0.05$; b vs Febrile controls $P < 0.01$; c vs Non2CALs KD group $P < 0.05$

MMP29 enzyme activity

1) The activity and levels of MMP29 in KD pa2 tients with CALs in the acute phase were significantly higher than those without ($P < 0.05$). Both of them in either the KD patients with CALs or without were higher than the febrile controls ($P < 0.01$) and normal controls ($P < 0.05$). 2) There was a signifi2 cantly positive correlation between the serum MMP29 levels and the circulating leucocytes counts in the a2 cute phase of KD ($r = 0.480$, $P < 0.05$). 3)

MMP29 mRNA expressions in the leucocytes of KD patients in the acute phase were significantly elevat2 ed, when compared with the febrile and normal con2 trols ($P < 0.01$). But there was no significant dif2 ference between the two KD groups. 4) The activity, protein levels and mRNA expressions of MMP29 of the KD patients decreased obviously from the suba2 cute through convalescent phases, as compared with the acute phase ($P < 0.01$). See Figure 1 and Table 2.

MMP29 total protein levels

A significant elevation of serum MMP29 protein levels was observed in 27 KD patients in the acute phase, compared with the subacute and the convales2 cent phases, as well as compared with the febrile and normal controls ($P < 0.01$). There were significant differences in the MMP29 protein levels in the acute phase between the two KD groups ($P < 0.05$). Furthermore, MMP29 protein levels in KD patients in the acute phase showed a significant correlation with the WBC counts ($r = 0.480$, $P < 0.05$) and the neutrophil counts ($r = 0.501$, $P < 0.01$), but not with the monocyte counts ($r = 0.089$, $P = 0.664$) (Table 2).

Figure 1 MMP29 activity in various groups

Note: Lands 123 show MMP29 activity in KD patients with CALs in the acute, subacute, convalescent phases respectively; Lands 426 show MMP29 activity in KD patients without CALs in the acute, suba2 cute, convalescent phases respectively; Lands 728 show MMP29 activity in the normal and febrile controls.

Table 2 Activity, protein levels and mRNA expression of MMP29 in various groups ($\bar{x} \pm s$)

Group	n	MMP29 activity (density)	MMP29 protein (ng/ml)	MMP29 mRNA (MMP29/β2actin)
Normal controls	10	0.1 ±0.0	72 ±24	0.19 ±0.09
Febrile controls	10	3.1 ±1.4[a]	221 ±154[a]	0.64 ±0.23[a]
KD	27			
Non2CALs	10			
Acute		6.2 ±2.1[a,b]	457 ±133[a,b]	0.88 ±0.14[a,b]
Subacute		3.0 ±1.8[d]	135 ±10[d]	0.60 ±0.13[d]
Convalescent		0.1 ±0.1[e]	75 ±36[e]	0.24 ±0.08[e]
CALs	17			
Acute		10.2 ±2.2[a,b,c]	1146 ±691[a,b,c]	0.94 ±0.16[a,b]
Subacute		3.1 ±1.2[d]	205 ±145[d]	0.60 ±0.18[d]
Convalescent		0.1 ±0.0[e]	81 ±40[e]	0.23 ±0.09[e]

Note: a vs Normal controls $P < 0.01$; b vs Febrile controls $P < 0.05$; c vs Non2CALs KD group $P < 0.05$; d vs the acute phase from the same group $P < 0.01$; e vs the subacute phase from the same group $P < 0.01$

MMP29 mRNA expression

No significant difference was observed in the a2 cute phase between the two KD groups for MMP29 mRNA expressions in circulating leucocytes. The MMP29 mRNA expressions in both KD groups in this phase were significantly higher than those in the febrile and normal controls ($P < 0.01$), although they decreased significantly ($P < 0.01$) from the subacute through convalescent phases (Figure 2).

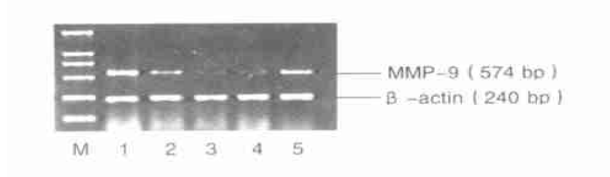

Figure 2 MMP29 mRNA expressions in circulating leucocytes

Note: Lands 123 stand for the acute, subacute, convalescent phases of KD respectively; Lands 425 stand for the normal and febrile controls respectively; M: stands for DNA marker (2 000 bp, 1 000 bp, 750 bp, 500 bp, 250 bp, 100 bp)

Discussion

The coronary arteries in children with acute KD are characterized by transmural inflammation and al2 terations of the extracellular matrix, with thinning of the vascular media and marked destruction of the in2 ternal elastic lamina, which lead to dilation or a2 neurysm formation. The mechanisms underlying de2 struction of elastin and other components of extracellular matrix in acute KD remain unknown. Matrix metallopro2 teinases (MMPs) play an important role in the progres2 sion of tumor cells and the invasion of inflammatory cells by degrading extracellular matrix. In the MMP family, MMP29 is thought to be involved in the pathogenesis of inflammatory arteritis by degrading the elastic lamina, such as temporal arteritis[5]. At the 7th International KD Symposium in December 2001, the importance of MMP2 9 in KD was put forward.

Recently, several researchers have independently reported elevated levels of MMP29 in the serum or plasma during the acute phase of KD patients[6-9], and thought MMP29 may be involved in the forma2 tion of coronary artery lesions. This study is the first in China to demonstrate significantly elevated MMP29 enzyme activity and protein levels in KD during the a2 cute phase, especially in KD with CALs. These find2 ings suggest that over2expressions of MMP29 may play a central role in the pathogenesis of coronary artery lesions in KD.

This study also showed there was a positive cor2 relation between MMP29 protein levels and the circu2 lating leukocytes counts in KD during the acute phase. By using RT2PCR, it was further demonstrat2 ed that the kinetics of the MMP29 mRNA expression in the circulating leukocytes of all acute KD was simi2 lar to the MMP29 protein levels. These findings are consistent with Takeshita's[8]. Chua[9] has ever found that peripheral blood monocytes, obtained from KD patients in the acute phase and cultivated in vit2 ro, can produce high levels of MMP29, which sug2 gests that immune cells, such as activated neutrophil and monocyte/macrophages, are the main source of MMP29 in circulation of KD patients. MMP29 secret2 ed from leucocytes is thought to be involved in facili2 tating extravasation and migration of these cells by breaking down the basement membrance[10]. In this study, circulating leukocytes counts in KD patients with CALs during the acute phase were significantly higher than those without CALs, which may inter2 pret why there was a significantly higher expression of MMP29 in the KD patients with CALs than those without. Activation of the immune system is a central feature of KD, and infiltrated inflammatory cells and pro2inflammatory cytokines, including TNF2α, IL21, IL26 and IFN2γ, are elevated in the acute phase of KD, yielding an increase of MMP29 expression[11].

Gavin[12] demonstrated that MMP29 was widely expressed in the arterial wall with coronary artery le2 sions in KD patients who died from cardiovascular complications, and it was not expressed in normal control coronary arteries, which presents a direct proof of MMP29 involvement in the formation of coronary artery lesions. Interestingly, the tissue in2 hibitors of metalloproteinase21 (TIMP21), an impor2 tant inhibitor of MMP29, were not expressed in the coronary arteries of KD patients - even in the pres2 ence of increased MMP29 expression[12]. Therefore, an imbalance between MMP29 and TIMP21 could ac2 count for overproduction of MMP29 in the inflamed coronary artery of acute KD, and lead to further di2 latation or aneurysm formation.

In summary, there was a remarkably elevated expression of MMP29 in KD patients during the acute phase, especially in those with CALs, which suggests that MMP29 may play an important role in the devel2 opment of coronary artery lesions. The pathogenesis and the regulation mechanism of MMP29 expression need to be studied in detail.

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·消息·

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