Percutaneous Transluminal Mitral Valvuloplasty Reduces Circulating Soluble CD40 Ligand in Rheumatic Mitral Stenosis*

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**Background:** Recent data suggest that the pathogenesis of vascular inflammation and thrombosis involves CD40 ligand (CD40L), which is mostly derived from platelets. Previous studies have demonstrated that platelet activation occurs in peripheral blood of patients with rheumatic mitral stenosis (MS). However, in patients with MS, the plasma level of soluble CD40L has never been investigated.

**Methods and results:** Seventeen patients with symptomatic MS undergoing percutaneous transluminal mitral valvuloplasty were studied (group 1, 11 patients in permanent atrial fibrillation and 6 patients in sinus rhythm). Solid-phase, sandwich enzyme-linked immunosorbent assay determined the plasma levels of soluble CD40L in the femoral vein and artery, and right and left atria before valvuloplasty, and those in the peripheral venous blood obtained 10 min after valvuloplasty, and at the 4-week follow-up after valvuloplasty. The Doppler pressure half-time method was used to calculate the mitral valve area. Additionally, plasma concentrations of soluble CD40L in the peripheral venous blood obtained from 17 control patients were measured (including nine healthy volunteers in sinus rhythm [group 2] and eight patients in permanent lone atrial fibrillation [group 3]). Plasma levels of soluble CD40L were significantly elevated in group 1 patients (437.6 ± 370.2 pg/mL) [mean ± SD] compared with group 2 (203.8 ± 218.0 pg/mL) and group 3 patients (173.5 ± 105.0 pg/mL) [p < 0.05]. The area of mitral valve increased significantly after valvuloplasty (1.10 ± 0.20 cm² vs 1.47 ± 0.29 cm², p < 0.0001). The mean left atrial pressure fell significantly and immediately after valvuloplasty (22.8 ± 4.9 mm Hg vs 17.6 ± 5.5 mm Hg, p = 0.0004). The peripheral venous plasma levels of soluble CD40L obtained before valvuloplasty significantly fell after valvuloplasty (before, 437.6 ± 370.2 pg/mL; vs 10 min after, 215.4 ± 113.9 pg/mL; vs 4 weeks after, 217.5 ± 111.9 pg/mL; p < 0.02).

**Conclusions:** Patients with moderate-to-severe MS had higher venous plasma levels of soluble CD40L than healthy volunteers or patients with lone atrial fibrillation. Additionally, the elevated venous plasma levels of soluble CD40L fell significantly following valvuloplasty.

(CHEST 2005; 128:36–41)

**Key words:** CD40 ligand; mitral stenosis; mitral valvuloplasty

**Abbreviations:** CD40L = CD40 ligand; MS = mitral stenosis; PTMV = percutaneous transluminal mitral valvuloplasty

S oluble CD40 ligand (CD40L) [CD154] is a 33-kd type II membrane glycoprotein that was first described as a surface marker exclusive to activated CD4⁺ T cells.¹⁻⁴ Other cell types including dendritic cells, B cells, and platelets have expressed CD40L.⁵⁻⁷ Ninety percent of the circulating soluble CD40L is derived from platelets. Soluble CD40L is

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Financial support was provided by research grant CMRP8037 from the Chang Gung Memorial Hospital, Chang Gung University, Taiwan.

Manuscript received October 14, 2004; revision accepted January 10, 2005. Reproduction of this article is prohibited without written permission from the American College of Chest Physicians (www.chestjournal.org/misc/reprints.shtml).

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released from membrane-bound CD40L as a degradation product, on platelet activation. CD40L belongs to the tumor necrosis factor family of cytokines. Studies\textsuperscript{8–11} suggest that CD40L, interplaying with CD40 or glycoprotein IIb/IIIa, is involved in the pathogenesis of vascular inflammation and thrombosis.

Systemic, especially cerebral, thromboembolism has been shown to be increased in patients with rheumatic mitral stenosis (MS) and is one of the major causes of illness and death in patients with MS.\textsuperscript{12,13} Previous studies\textsuperscript{14,15} have demonstrated that platelet activation occurs in peripheral blood of patients with rheumatic MS. Chen et al\textsuperscript{16} demonstrated that in patients with moderate-to-severe rheumatic MS, increased regional left atrial platelet P-selectin (or CD 62p antigen, a biologically relevant molecule that is released to the surface of platelet from α-granules on activation) expression has a significantly direct relationship with the severity of MS. Additionally, the increased regional left atrial platelet P-selectin expression is not reflected in peripheral venous blood samples. Chen et al\textsuperscript{17} also demonstrated that in patients with moderate-to-severe MS, increased platelet activation falls significantly after percutaneous transluminal mitral valvuloplasty (PTMV). No previous study has investigated the circulating levels of soluble CD40L in patients with rheumatic MS. A hypothesis was made that in patients with rheumatic MS, plasma levels of soluble CD40L are elevated and the elevated plasma soluble CD40L levels might reduce following PTMV. Accordingly, the present study investigates plasma levels of soluble CD40L in patients with rheumatic MS.

**Materials and Methods**

**Study Population**

Seventeen patients with symptomatic, moderate-to-severe rheumatic MS (mitral valve area, 1.1 ± 0.2 cm\textsuperscript{2} [± SD]; range, 0.71 to 1.58 cm\textsuperscript{2}) without significant mitral, tricuspid, or aortic regurgitation, or left atrial thrombus, who underwent PTMV were studied (group 1). Criteria for diagnosis of rheumatic MS included the presence of commissional fusion, leaflet thickening, and alteration of the subvalvular apparatus documented by echocardiogram.\textsuperscript{13} All of the patients were women (age range, 33 to 72 years; mean, 53 ± 14 years). Eleven patients had permanent atrial fibrillation, and 6 patients were in sinus rhythm. Four patients had a history of cerebral thromboembolism. Seven patients were in New York Heart Association functional class III, and 10 patients were in New York Heart Association functional class II. No patients had a history of malignancy, inflammatory disease, collagen vascular disease, renal or liver disease, diabetes mellitus, hypertension, hyperlipidemia, infectious disease, deep venous thrombosis, pulmonary embolism, or recent surgery.

Peripheral venous plasma levels of soluble CD40L were also measured in 17 control patients. The group of control subjects included nine healthy volunteers in sinus rhythm (group 2) and eight patients in permanent lone atrial fibrillation without systemic disease or structural heart disease (group 3). In group 3, two patients had a history of systemic arterial thromboembolism. None of the control patients had a history of active malignancy, inflammatory disease, renal or liver disease, diabetes mellitus, hypertension, hyperlipidemia, deep venous thrombosis, pulmonary embolism, or recent surgery.

Informed consent was obtained from all study subjects. The study protocol was approved by the Institutional Review Committee on Human Research in our institution.

**Doppler Echocardiography and Medications**

In patients with rheumatic MS, transthoracic echocardiography was performed on the day of PTMV and before valvuloplasty with a 2.5-MHz transducer attached to a commercially available Doppler echocardiographic device (Sonos 5500; Hewlett-Packard; Palo Alto, CA) to measure left and right atrial dimensions, and mitral valve area. M-mode measurements were performed according to the recommendation of the American Society of Echocardiography. Left and right atrial areas were planimetered in the four-chamber view, and maximum areas were measured (at the end of the T wave on the ECG) and averaged over five beats. Doppler pressure half-time method calculated the mitral valve area. Transesophageal echocardiography confirmed the absence of a left atrial cavity or appendage thrombus. All patients had swirling spontaneous echo contrast only in the left atrium confirmed by transesophageal echocardiography.

In group 1 patients, digoxin, β-blockade, and calcium-channel blockade were discontinued for at least five half-lives before study, and therapy with diuretic agents was discontinued on the day of PTMV. Warfarin therapy was discontinued for 3 to 4 days before the patients underwent PTMV and was administered on the second day after PTMV. During the follow-up studies, warfarin therapy was also discontinued for 3 to 4 days before blood examinations. Heparin, 5,000 U, was administered into the left atrium after transseptal puncture in each patient. In group 3 patients, therapy with aspirin was discontinued for at least 7 days, digoxin and calcium-channel blockade were discontinued for at least five half-lives before study, and warfarin was discontinued for 3 to 4 days before the study.

**Valvuloplasty Procedure**

PTMV was performed by the transseptal approach with the use of an Inoue balloon catheter (Toray Medical Corporation; Tokyo, Japan). Details of the procedure have been described previously.\textsuperscript{18} Measurements of left and right atrial pressure, pulmonary artery pressure, and transmitral pressure gradient were performed immediately before and after valvuloplasty.

**Blood Sample Collection and Measurement of Plasma Soluble CD40L Concentrations**

Blood samples were obtained in the fasting, non-sedative state at 9 to 10 AM in the control and study groups to exclude the possible influence of circadian variations. In group 1 patients, blood was obtained from the femoral vein and artery through introducer sheaths immediately after puncture with the patients in the supine position for at least 20 min. Right atrial blood was obtained through the balloon catheter, and left atrial blood was obtained immediately after transseptal puncture before heparin administration. Another set of blood samples from the femoral vein, femoral artery, and right and left atria were obtained 10 min after optimal PTMV. At the 4-week follow-up after PTMV, peripheral venous blood was obtained under minimal tourniquet.
pressure from the antecubital vein using a sterile 22-gauge needle syringe in a single attempt with the study patients in the supine position for at least 20 min. Five milliliters of blood was drawn into an evacuated tube containing K3 ethylenediamine tetra-acetic acid (Vacutainer; Becton Dickinson; Franklin Lakes, NJ). In groups 2 and 3 subjects, blood was also obtained from the antecubital vein. Blood samples with gross hemolysis were discarded. Mixtures of blood and K3 ethylenediamine tetracetic acid (Vacutainer; Becton Dickinson; Franklin Lakes, NJ). The samples were processed according to the instructions of the manufacturer. The samples, which included standards of known recombinant human CD40L concentrations in buffer and test samples and an enzyme (horse-radish peroxidase)-labeled second antibody, were sequentially added to a 96-well microplate precoated with a polyclonal antibody specific for human CD40L. After 2 h of incubation at room temperature and removal of unbound materials, the amount of enzyme-conjugated tracer bound to the wells was detected through reaction with a substrate (tetramethylbenzidine) specific for the enzyme. A microplate reader (MRX; Dynex Technologies; Chantilly, VA) was used to measure the reaction product and reading the absorbance at 450 nm with a correction wavelength of 550 nm. The mean absorbance values of the included CD40L standards determined a standard curve, and the soluble CD40L concentrations in all unknown plasma samples was then calculated with linear regression. All standards and samples were tested in duplicate. In this study, the minimum detectable dose of CD40L was 10 pg/mL. The mean intra-assay coefficient of variance of 40 plasma samples was 2.9%.

Statistical Analysis

Continuous variables were described as the mean ± SD. Categorical variables were compared using the Fisher Exact Test (two-tailed). To improve the normality for statistical analysis, log-transformation of plasma soluble CD40L concentrations was utilized. Continuous variables within the same group were compared using Wilcoxon paired rank-sum test. Continuous variables among the three groups were compared using the Kruskal-Wallis test. Multiple logistic regression analysis was used to determine the significance of dependent variable among multiple independent variables. The plasma levels of soluble CD40L of the four different sampling sites were compared using the repeated-measures of analysis of variance. The plasma levels of soluble CD40L in the peripheral venous blood obtained before PTMV, 10 min after PTMV and at the 4-week follow-up were compared using the repeated-measures of analysis of variance. Post hoc comparisons utilized the Tukey multiple comparison procedure. The correlation between the plasma level of soluble CD40L and mitral valve area, the mean left atrial and pulmonary artery pressures, or left atrial dimension were performed with the Spearman correlation. Statistical analysis was performed using a statistical software program (SAS for Windows, Version 8.02; SAS Institute; Cary, NC); p < 0.05 was statistically significant.

Results

Comparison of Baseline Characteristics and Peripheral Venous Plasma CD40L Concentrations Among the Three Groups

The baseline characteristics for each group are summarized in Table 1. No statistically significant differences among the three groups in terms of blood cell counts and biochemical data were found. No differences were found among the three groups in terms of the use of β-blockade (group 1, 29.4%; vs group 2, 0.0%; vs group 3, 0.0%), calcium-channel blockade (group 1, 29.4%; vs group 2, 0.0%; vs group 3, 12.5%), amiodarone (group 1, 5.9%; vs group 2, 0.0%; vs group 3, 0.0%), propafenone (group 1, 5.9%; vs group 2, 0.0% vs. group 3, 0.0%), and aspirin (group 1, 0.0%; vs group 2, 0.0%; vs group 3, 12.5%). The use of warfarin therapy in patients in group 1 (82.4%) and group 3 (87.5%) was more frequent than in patients in group 2 (0.0%) because of atrial fibrillation. The use of digoxin in patients from group 1 (47.1%) was more frequent than that in groups 2 (0.0%) and 3 (25.0%). The duration of atrial fibrillation of group 1 patients did not differ from that of group 3 patients. Group 3 patients were

| Table 1—Baseline Characteristics and Peripheral Venous Plasma CD40L Concentrations of Patients Studied* |
| Variables | Group 1 (n = 17) | Group 2 (n = 9) | Group 3 (n = 8) |
| Age, yr | 53 ± 14 | 39 ± 11 | 66 ± 14† |
| Women, % | 100.0‡ | 66.7 | 37.5 |
| Duration of atrial fibrillation, mo | 44.9 ± 58.1 | 0.0 | 33.8 ± 35.9 |
| History of embolism, % | 23.5 | 0.0 | 25.0 |
| Platelets, 10^4 cells/μL | 198.8 ± 5.7 | 253.4 ± 4.1 | 207.4 ± 8.0 |
| Hemoglobin, g/dL | 11.6 ± 1.6 | 13.1 ± 2.6 | 13.0 ± 2.6 |
| Leukocytes, 10^3 cells/μL | 6.3 ± 2.3 | 6.2 ± 1.6 | 5.9 ± 2.5 |
| Calcium, mg/dL | 8.7 ± 0.4 | 8.9 ± 0.4 | 9.2 ± 0.6 |
| CD40L in the peripheral venous plasma, pg/mL | 437.6 ± 370.2§ | 203.8 ± 218.0 | 173.5 ± 105.0 |

*Values given as mean ± SD or % of patients.
†p = 0.006, group 2 vs group 3.
‡p < 0.001, group 1 vs groups 2 and 3.
§p < 0.05, group 1 vs groups 2 and 3, Kruskal-Wallis test.
significantly older than were group 2 patients. There were more women in group 1 than in groups 2 and 3. The peripheral venous plasma levels of soluble CD40L were significantly higher in group 1 patients than in group 2 or 3 patients (p < 0.05), even after adjustment for multiple independent variables. The peripheral venous plasma levels of soluble CD40L among group 2 subjects did not differ from those among group 3 patients.

Comparison of Plasma Levels of Soluble CD40L Among the Four Different Sampling Sites

Analysis of the prevalvuloplasty data of the 17 patients with rheumatic MS revealed that the plasma levels of soluble CD40L in the left atrium (1,486.1 ± 2,319.3 pg/mL) were borderline significantly higher than those in the right atrium (386.2 ± 388.2 pg/mL) [p = 0.0495]. The plasma levels of soluble CD40L in the left atrium (1,486.1 ± 2,319.3 pg/mL) were not significantly higher than those in the femoral vein (437.6 ± 370.2 pg/mL) or femoral artery (748.0 ± 1,504.1 pg/mL). The plasma levels of soluble CD40L in the right atrium did not significantly differ from those in the femoral vein or femoral artery.

Correlation Between Plasma Levels of Soluble CD40L and Hemodynamic and Echocardiographic Variables in Patients With Mitral Stenosis

Correlation analysis demonstrated that no significant association existed between the plasma levels of CD40L in the left atrium and left atrial diameter (p = not significant; r = -0.036), left atrial area (p = not significant; r = 0.130), prevalvuloplasty mean left atrial pressure (p = not significant; r = 0.332), prevalvuloplasty mean pulmonary artery pressure (p = not significant; r = 0.236), and the severity of MS (p = not significant; r = -0.188).

Peripheral Venous Plasma Levels of Soluble CD40L Before and After Mitral Valvuloplasty

No significant changes were found in left atrial diameter, left and right atrial areas, left ventricular dimension, and ejection fraction following PTMV (Table 2). Mitral valve area significantly increased after PTMV (p < 0.0001). Mean left atrial pressure fell significantly and immediately following PTMV (p = 0.0004). The transmitral pressure gradient significantly fell after PTMV (p = 0.0006). Peripheral venous plasma levels of soluble CD40L obtained before PTMV significantly fell after PTMV (before, 437.6 ± 370.2 pg/mL; vs 10 min after, 215.4 ± 113.9 pg/mL; vs 4 weeks after, 217.5 ± 111.9 pg/mL; p < 0.02) [Fig 1].

**DISCUSSION**

The present study examined the plasma soluble CD40L concentrations in atrial and peripheral venous or arterial blood samples of patients with symptomatic rheumatic MS undergoing PTMV, and produced two main findings. First, patients with moderate-to-severe MS had higher venous plasma levels of soluble CD40L than healthy volunteers or patients with lone atrial fibrillation. Secondly, the elevated venous plasma levels of soluble CD40L significantly fell after PTMV.

**Increased CD40L Concentrations in Patients With MS**

Previous studies demonstrated that platelet activation occurs in peripheral blood of patients with rheumatic MS. Platelets appear to be the predominant source of circulating soluble CD40L. This study demonstrates that venous plasma soluble CD40L levels were elevated in patients with moderate-to-severe MS. Soluble CD40L is prothrom-

| Table 2—Echocardiographic and Hemodynamic Variables Before and After PTMV* |
|-----------------|-----------------|-----------------|-----------------|
| Variables       | Before PTMV     | After PTMV      | p Value         |
| Left atrial diameter, mm | 46.0 ± 6.8      | 45.2 ± 7.5      | 0.21            |
| Left atrial area, cm²  | 35.9 ± 11.6     | 33.6 ± 13.4     | 0.16            |
| Right atrial area, cm² | 21.2 ± 8.9      | 20.7 ± 8.5      | 0.55            |
| Left ventricular end-diastolic diameter, mm | 44.6 ± 6.0      | 45.2 ± 5.2      | 0.23            |
| Left ventricular end-systolic diameter, mm | 28.8 ± 4.3      | 27.6 ± 4.7      | 0.23            |
| Ejection fraction, %  | 63.7 ± 9.9      | 68.5 ± 8.4      | 0.16            |
| Mitral valve area, cm² | 1.10 ± 0.20     | 1.47 ± 0.29     | < 0.0001        |
| Mean left atrial pressure, mm Hg | 22.8 ± 4.9      | 17.6 ± 5.5      | 0.0004          |
| Transmitral pressure gradient, mm Hg | 11.0 ± 5.1      | 7.2 ± 4.4       | 0.0006          |
| Mean pulmonary artery pressure, mm Hg | 30.3 ± 7.6      | 24.5 ± 7.3      | 0.0004          |
| Mean aortic pressure, mm Hg | 97.2 ± 12.5     | 93.9 ± 12.7     | 0.097           |

*Data presented are mean ± SD.

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bolic via stabilization of arterial thrombi by a $\beta_3$-integrin (glycoprotein IIIa)-dependent mechanism and is proinflammatory via induction of leukocyte chemokine production and endothelial cell adhesive protein. High plasma concentrations of soluble CD40L may be associated with increased cardiovascular risk in apparently healthy women. Whether the elevated circulating soluble CD40L in patients with moderate-to-severe MS contributed to the increased risk of systemic embolism is unknown.

Although the use of warfarin and digoxin therapies in patients with MS was more frequent than that in healthy volunteers and more female patients with MS compared with healthy volunteers in this study, in the multivariate model (multiple logistic regression analysis), the peripheral venous plasma levels of soluble CD40L were independently and significantly higher in patients with MS than in healthy volunteers.

Chen et al demonstrated that in patients with moderate-to-severe MS, the fraction of platelets expressing P-selectin (platelet activity) in the left atrium was significantly higher than that in the right atrium, femoral vein, or femoral artery. As platelets appear to be the predominant source of circulating soluble CD40L, it is reasonable to observe higher plasma levels of CD40L in the left atrium compared to the plasma levels of CD40L in the blood drawn from sites distal to the valve, such as the femoral artery and vein, and right atrium. Chen et al also demonstrated that increased regional left atrial platelet P-selectin expression had a significantly direct relationship with the severity of MS. In the present study, no significant association existed between the plasma levels of CD40L in the left atrium and the severity of MS. These findings indicate that plasma soluble CD40L levels could not reflect changes in individual platelets.

Reducing CD40L Following PTMV

Platelets appear to be the predominant source of circulating soluble CD40L. CD40L is not expressed on the resting platelet surface. Platelet activation is required for the release of soluble CD40L. Platelets translocated preformed intraplatelet stores of CD40L to the platelet surface within seconds of activation in vitro. Additionally, platelet activation results in fibrinogen binding to the glycoprotein IIb/IIIa complex, which results in activation of the cytoskeleton via actin polymerization, movement of the protease (metalloproteinases) to the platelet surface, and therefore release of soluble CD40L, after which soluble CD40L levels decreased over time with an approximate half-life of 6 h in vitro. Natural soluble CD40L is relatively unstable. Additionally, the metabolic pathway of soluble CD40L remains unknown. Therefore, the half-life of soluble CD40L in vivo remains unclear. Furman et al demonstrated that both cytochalasin D (an inhibitor of actin polymerization) and GM6001 (an inhibitor of matrix metalloproteinases) inhibit the release of soluble CD40L from platelets when added before, as well as 3 min after, platelet activation. Additionally, Nannizzi-Alaimo et al showed that the release of soluble CD40L from activated platelets reduced as early as 5 min after incubating with epifibatide, a glycoprotein IIb/IIIa antagonist that inhibits platelet aggregation and activation. Evidence from several studies has shown that shear stresses in turbulent flow as a result of stenotic valves induce platelet activation. Chen et al demonstrated that in patients with moderate-to-severe MS, increased platelet activation significantly falls after PTMV, in accordance with the increase in mitral valve area after PTMV. Zaki et al showed that platelet activity immediately decreased after optimal PTMV results. We proposed that PTMV rapidly reduced platelet activity in patients with moderate-to-severe MS, consequently rapidly reducing soluble CD40L released from platelets. Therefore, it is reasonable to observe that venous plasma levels of CD40L rapidly decreased after PTMV.

Study Limitations

First, although warfarin therapy was discontinued for 3 to 4 days in patients in groups 1 and 3, the potential late effect of warfarin on platelet activity could not be concluded. However, the plasma levels of soluble CD40L in group 1 patients were significantly higher than those in groups 2 and 3, and the plasma levels of soluble CD40L in group 2 did not differ from those in group 3. These should exclude the potential late effect of warfarin on platelet activity, as most of the groups 1 and 3

**Figure 1.** The peripheral venous plasma levels of soluble CD40L obtained before valvuloplasty (PBb), 10 min after valvuloplasty (PBa), and at the 4-week follow-up after valvuloplasty (PB4w) in patients with rheumatic MS. The venous plasma levels of soluble CD40L obtained before valvuloplasty were significantly higher than those obtained after valvuloplasty; $*p < 0.02.
patients received warfarin before entering the study. Secondly, in the present study, the plasma levels of soluble CD40L among patients with lone atrial fibrillation did not differ from those of healthy volunteers. This finding should be viewed as preliminary and await confirmation by larger clinical study. Finally, as the number of patients having a history of systemic arterial thromboembolism was small, the aim of this work was not to study the difference in the plasma levels of soluble CD40L between patients with and without a history of systemic arterial thromboembolism.

In conclusion, the venous plasma levels of soluble CD40L in patients with moderate-to-severe MS were significantly higher than those in healthy volunteers or patients with lone atrial fibrillation. Additionally, the elevated venous plasma levels of soluble CD40L significantly fell after PTMV.

ACKNOWLEDGMENT: The authors thank Ms. Hsin-Chin Tsai for technical assistance.

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