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Relation between upregulation of CD40 system and complex stenosis morphology in patients with acute coronary syndrome[1]

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ABSTRACT

AIM: To investigate whether upregulation of CD40-CD40 ligand system is related to matrix metalloproteinases level and stability of coronary atherosclerotic plaque in patients with acute coronary syndrome (ACS). **METHODS:** Sixteen normal controls and 56 patients including 24 with stable angina (SA), 20 with unstable angina (UA), and 12 with acute myocardial infarction (AMI) were investigated. The expression of CD40 and CD40L on platelet was analyzed by flow cytometry. Serum soluble CD40L (sCD40L), MMP-9 and MMP-3 level was determined by ELISA. All coronary stenosis with ≥30 % diameter reduction were assessed by angiographic coronary stenosis morphology. **RESULTS:** Patients with ACS showed a significant increase of CD40 (75±12 MIF) and CD40L (13±4 MIF) coexpression on platelets compared with control and SA group (P<0.01). sCD40L also showed higher level in patients with ACS (10.2±3.5 μg/L) than in control (3.1±1.4 μg/L, P<0.01) and SA group (3.3±1.6 μg/L, P<0.01). Serum MMP-3 and MMP-9 in patients with ACS were two times greater than those in control. A positive correlation was found between MMP-9, MMP-3, and CD40L expression on platelets as well as sCD40L levels, but not for CD40 expression on platelets. An obvious correlation was also observed between sCD40L concentration and complex coronary stenoses (r=0.60, P<0.01). **CONCLUSION:** Patients with ACS show increased coexpression of CD40 system, especially expression of CD40L, which may create a proinflammatory and prothrombotic milieu for aggravating the development of atherosclerosis and instability of atherosclerotic plaques, and may be a valuable marker for predicting the severity of ACS.

INTRODUCTION

Disruption of vulnerable atheromatous plaque is the most common pathogenic mechanism in acute coronary syndromes (including non Q wave AMI, Q wave AMI and unstable angina). Integrity of the extracellular matrix constitutes a critical determinant in the stability of coronary atheromata. In particular, degradation of fibrillar collagen may decrease the ability of the fibrous cap to withstand mechanical stress. Several members of the MMP family contribute to collagen degradation: interstitical collagenase (MMP-1), stromelysin (MMP-3), and gelatinase B (MMP-9). MMPs play a pathogenic role in the development of ACS. Studies have recently supported the emerging role of CD40-CD40 ligand (CD40L or CD154) interaction in atherosclerosis, thrombosis, and inflammation[1]. CD40-CD40L inter-

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action mediates inflammatory responses that could serve to accelerate the atherosclerotic process and trigger plaque rupture.

Complex lesions are associated with rapid disease progression and a higher restesosis rate after percutaneous transluminal coronary angioplasty as compared with smooth lesions, probably reflecting a tendency toward thrombogenesis or further plaque disruption, or both.

Until now, little information has addressed the potential relationship between CD40 system and MMPs as well as coronary complex stenosis morphology in patients with ACS. Therefore, the present study was designed to investigate the coexpression of CD40 system on circulating platelets, serum soluble CD40L, and assess the correlation with serum MMP-3 and MMP-9 levels, as well as the number of complex lesions in the patients.

Tab 1. Characteristics of the study groups.

Groups	Control (n=16)	SA (n=24)	UA (n=20)	AMI (n=12)
Age, a	51±10	61±8	59±11	63±10
Sex, M/F	12/4	18/6	13/7	9/3
Total cholesterol, mmol/L	4.9±0.8	5.0±1.0	5.1±0.9	4.8±1.0
HDL,cholesterol mmol/L	1.2±0.5	1.0±0.4	1.1±0.7	0.9±0.3
Triglycerides, mmol/L	1.5±0.7	1.7±0.5	1.6±0.9	1.8±1.0
Medication, %				
Calcium antagonist	0	9	11	13
Nitroglycerin	0	13	21	19
ACEI	0	9	8	12
β-blockers	0	82	78	80
HMG-COA reductase inhibitors	0	61	64	67
Aspirin	0	90	93	92

MATERIALS AND METHODS

Reagents Mouse-anti-human-CD40, mouse-anti-human-CD40L, CD61-FITC, and mouse IgG-PE-conjugated were bought from PharMingen. sCD40L ELISA kit was from Bender Medsystems. MMP-3 ELISA kit was obtained from Chemicon International Inc (systems detection limit, 0.25 μg/L) and MMP-9 kit was from R&D systems (detection limit, 0.156 μg/L).

Patients and control Patients undergoing clinically indicated diagnostic coronary angiography in our coronary care unit were consecutively registered. Twenty patients with unstable angina (Braunwald class III) had experienced ischemic chest pain at rest within the preceding 48 h, but they had no evidence of myocardial necrosis by enzymatic criteria, 24 patients with stable angina underwent coronary angiography because of signs and symptoms of clinically stable angina. Twelve patients with AMI<6 h after the onset of symptoms. Inclusion criteria were typical chest pain and ST segment elevation in at least two contiguous electrocardiographic leads. For comparison 16 sex- and age-matched donors served as a control group. All patients with infection, tumor, liver or kidney diseases were excluded (Tab 1).

Blood sampling protocol Peripheral venous blood was drawn into blood collection tubes containing sodium citrate. Citrated blood samples were either centrifuged (200×g at room temperature for 10 min) to obtain platelet rich-plasma or immediately fixed with 1 % formaldehyde (1:1, v:v). Noncitrated blood was immersed in melting ice and allowed to clot for 1 h before centrifugation (1500×g at 4ºC for 10 min). The supernatant was stored at -80 ºC until analysis. Samples were thawed only once.

Detection of CD40 and CD40L on platelets by flow cytometry Platelet immunostaining was performed as previously described[2]. Fixed blood was diluted 1:100 with PBS and incubated with the first antibody (30 min, 4 ºC), dilution with PBS. Then platelets were incubated with PE-conjugated second antibody (30 min, 4 ºC) and analyzed using CELLQUEST software. For each treatment the mean fluorescence intensity (MFI) value for the control stained population was subtracted from the MFI value of the positive-stained sample. Platelets were identified by gating on CD61-FITC positivity and their characteristic light scatter. The platelet population evaluated was ≥98 % positive for CD61.

Detection of sCD40L by ELISA Levels of sCD40L were determined by ELISA (sCD40 detection limit, 95 ng/L; Bender Medsystems) according to the manufacturer's instructions.

MMP-9 and MMP-3 measurements Serum levels of MMP-3 and MMP-9 were determined by ELISA according to the manufacturer's instructions.

Angiographic coronary stenosis morphology All coronary stenoses with ≥30 % diameter reduction were assessed by two experienced cardiologists who had no knowledge of the serum soluble CD40L results or the identity and clinical characteristics of the patients. Stenosis morphology was assessed as reported previ-

ously[3]. Briefly, stenoses were considered to be complex or smooth. Complex lesions were defined by the following features: 1) irregular morphology or scalloped borders, or both; 2) overhanging or abrupt edges perpendicular to the vessel wall; 3) ulceration; and/or 4) the presence of filling defects consistent with intracoronary thrombus. Coronary stenoses without complex features were classified as smooth lesions.

Statistical analysis Statistical evaluation was performed with Prism3.0 and SAS 7.0 software. Data were expressed as mean±SD and compared by unpaired *t*-test and ANOVA. Correlation was evaluated using regressive analysis. The Spearman two-way test was used to assess the relation between two quantitative variables with non-normal distribution. The Pearson two-way test was used to assess the relation between two quantitative variables with normal distributions. $P<0.05$ was considered statistically significant.

RESULTS

CD40 and CD40L expression on platelets CD40 and CD40L expression on platelets were significantly increased in patients with ACS compared with those obtained from control (CD40: 75±12 *vs* 47±11 MFI; CD40L: 13±4 *vs* 4.5±1.5 MFI; all $P<0.01$; Fig 1A). The increased expression of CD40L on platelets from ACS patients showed a positive correlation with serum levels of MMP-9 and MMP-3 ($r_1=0.53$, $P=0.002$; $r_2=0.56$, $P=0.0008$. $n=32$, Fig 1B). In contrast, no correlations were found between CD40 expression on platelets in patients and serum levels of MMP-9 and MMP-3 ($r_1=0.21$, $P=0.24$; $r_2=0.27$, $P=0.14$, $n=32$, respectively).

Serum MMP-3 and MMP-9 levels in patients with ACS Patients with ACS had more than twice the serum concentrations of MMP-3 and MMP-9 compared with controls and SA group. Serum MMP-3 (17.5±7.3 μg/L) and MMP-9 (31.2±10.7 μg/L) concentrations in patients with SA did not differ from controls (15.8±10.2, 28.9±15.7 μg/L, respectively, $P>0.05$, Fig 2).

Serum soluble CD40L and correlation of sCD40L with MMP-9, MMP-3 The level of sCD40L in patients with SA was not different from that in controls (3.3±1.6 *vs* 3.1±1.4 μg/L). However, the level of sCD40L was markedly elevated in the circulating blood of patients with UA (10.0±3.4 μg/L) or AMI (10.6±3.8 μg/L) compared with SA patients or with control. No

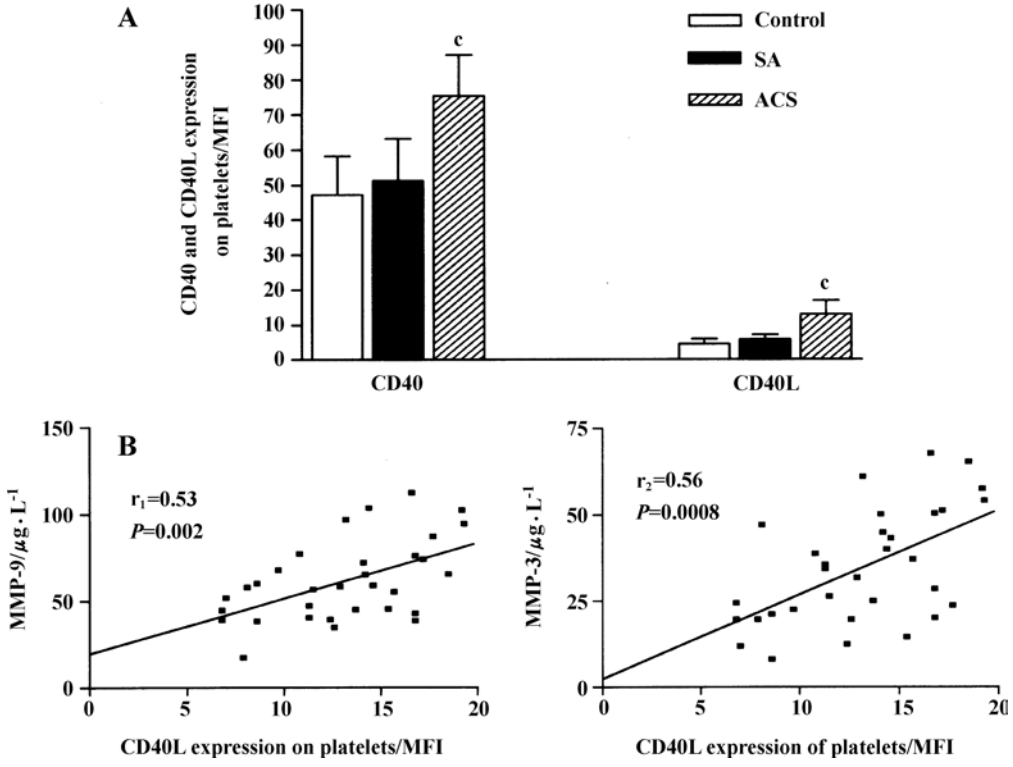

Fig 1. A) CD40 and CD40L expression on platelets in controls and the patients. $^cP<0.01$ *vs* control and SA group. B) Correlations between serum levels of MMP-9, MMP-3, and CD40L expression on platelets in patients with ACS.

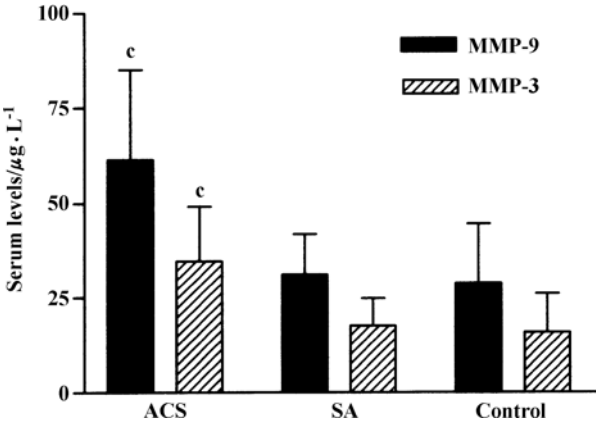

Fig 2. Comparison of MMP-3 and MMP-9 serum levels between patients with ACS and controls. $^{c}P<0.01$ *vs* SA group and control.

difference was found in the level of sCD40L between patients with UA and those with AMI (Fig 3). Furthermore, the correlation of sCD40L with serum levels of MMP-9 (n=32, r=0.43, P=0.01) or MMP-3 (n=32, r=0.47, P=0.006) was obviously observed in patients with ACS.

Correlation of serum soluble CD40L and complex coronary artery stenoses We observed a significant relation between sCD40L level and the number of complex lesions (r=0.60, P=0.0003, n=32). However, sCD40L levels did not correlate with number of smooth lesions (r=0.09, P=0.59, n=32) or stenosis severity (r=-0.08, P=0.63, n=32. Fig 4).

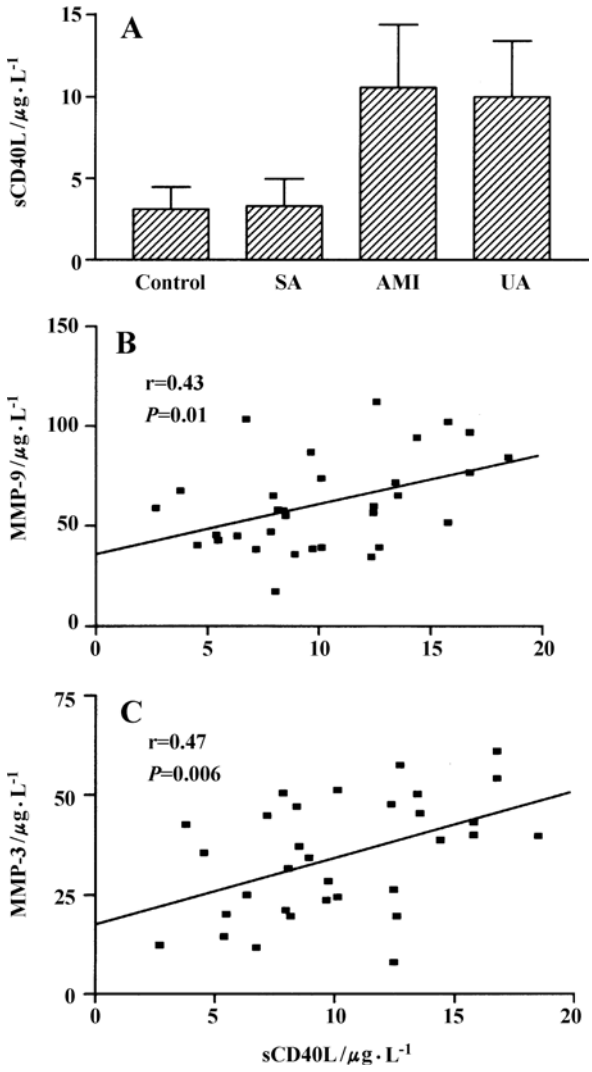

Fig 3. A) Serum soluble CD40L levels in patients with ACS and control. B) and C) The correlation of sCD40L with MMP-9 and MMP-3, there was a significant correlation of sCD40L with serum MMP-9 (r=0.43, P=0.01, n=32) and MMP-3 levels (r=0.47, P=0.006, n=32).

DISCUSSION

Acute coronary syndromes of unstable angina, acute myocardial infarction, and ischemic sudden death result from disruption of atherosclerotic plaque, leading to coronary thrombosis. The potential cellular mechanisms involved in plaque disruption are complex. In this study, we assessed, for the first time, the relation between CD40-CD40L system and the serum levels of matrix metalloproteinases (MMP-3, MMP-9), as well as angiographically demonstrated complex stenoses in patients with acute coronary syndrome.

Induction of MMP-9 expression as well as MMP-1 and MMP-2 has been shown in both vascular smooth muscle cells and accumulating macrophages in atherosclerotic plaques, particularly in the shoulder and core of plaques prone to rupture. These observations raised the possibility that these MMPs are strongly associated with the molecular mechanism of the onset and development of ACS. Synthesis of MMPs has also been reported in coronary atherosclerotic lesions in patients with unstable angina (UA) and AMI[4,5], which suggests a pathogenic role of MMPs in the development of ACS as well. In the current study, we demonstrated that both serum MMP-3 and serum MMP-9 in patients with ACS were increased compared with those in patients with SA and control subjects.

We and others reported that serum soluble CD40L was elevated in patients with ACS[6,7]. The present study showed that both CD40, CD40L expression on platelets and sCD40L were increased in patients with ACS. Regression analysis indicated that serum levels of MMP-3 and MMP-9, which degrade the connective tissue

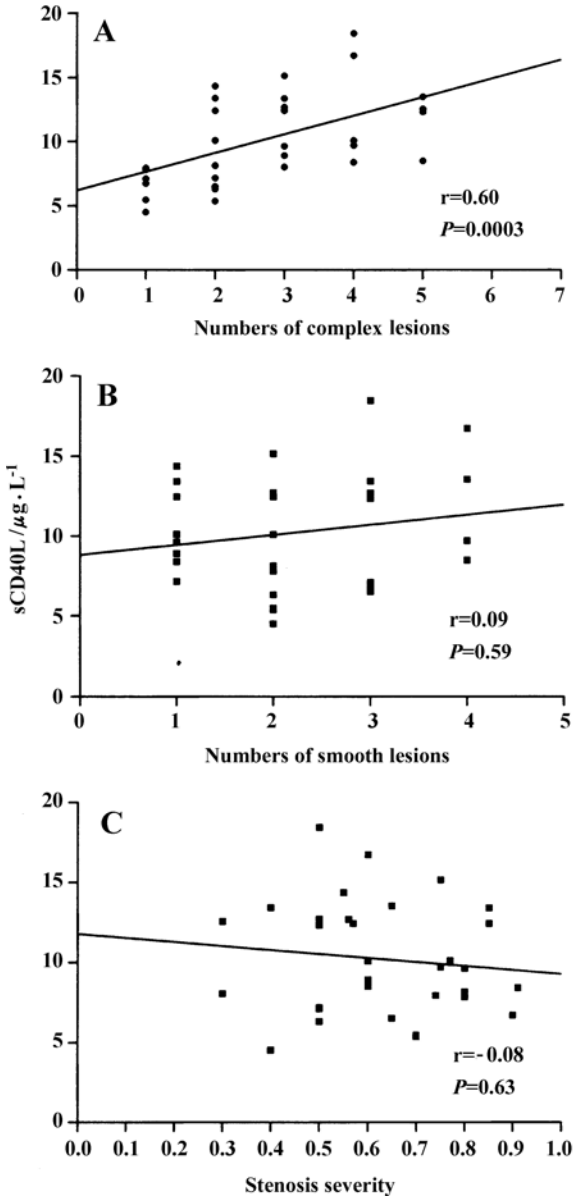

Fig 4. Correlations of serum soluble CD40L with numbers of complex lesions (A), numbers of smooth lesions (B) and stenosis severity (C), *n*=32.

matrix protein and ultimately lead to plaque rupture and the development of an acute coronary syndrome, remained significantly associated with CD40L levels on platelets and in serum. These findings suggested that CD40L expression in patients with ACS might be more important than CD40 expression, and might play a key role in the interaction of CD40-CD40L in ACS. Further investigations are needed to address these issues.

We believe that both increased membrane-bound CD40L and increased serum levels of sCD40L in patients with ACS not only are markers of immune activation, but also may be involved in pathogenic processes in these patients. sCD40L in circulation may pass through damaged atherosclerotic endothelium and come into direct contact with cells inside the lesion. However, even more importantly, sCD40L may activate circulating leukocytes or platelets to enhance the release of MMPs, increase the rupture of the plaques. Recently, many studies have demonstrated that the interaction of CD40-CD40L associated with the early formation of atherosclerosis and the long-term atherosclerotic process[8,9]. Moreover, experiment *in vitro*[10] demonstrated that administration of a neutralizing anti-CD40L monoclonal antibody could mitigate the development of atherosclerosis in mice.

Compared with patients with stable angina pectoris, those with unstable angina and AMI have a high number of complex coronary lesions[11]. It has been reported that the progression of these stenoses is faster than that of smooth stenoses. It has also been demonstrated that angiographically complex lesions represented vulnerable plaques prone to disruption or truly disrupted plaques[12,13]. In this study, we observed that there was a positive relation between sCD40L concentration and the number of complex lesions. In contrast, the significant correlation of sCD40L levels with number of smooth lesions (r=0.16, $P>0.05$) or stenosis severity (r=0.07, $P>0.05$), was not observed in the patients. Therefore, our findings suggest that CD40L may be a marker of coronary disease activity rather than a measure of the anatomic extent of coronary artery disease. Further, it supports the notion that sCD40L is a marker of plaque activity provided by a recent study of Aukrust *et al*[14].

In conclusion, the present study suggested that the membrane-binding CD40L and sCD40L serum concentration might be useful clinical markers of disease activity, and that therapeutical modalities by downregulating CD40-CD40L interaction may represent a new therapeutical approach in these patients.

REFERENCES

1 Phipps RP. Atherosclerosis: the emerging role of inflammmation and the CD40-CD40 ligand system. Proc Natl Acad Sci USA 2000; 97: 6930-2.
2 Michelson AD, Barnard MR, Krueger LA. Frelinger AL 3rd, Furman MI. Evaluation of platelet function by flow cytometry. Methods 2000; 21: 259-70.
3 Kaski JC, Chester MR, Chen L, Katritsis D. Rapid angiographic progression of coronary artery disease in patients with angina pectoris: the role of complex stenosis morphology. Circulation 1995; 92: 2058-65.
4 Kai H, Ikeda H, Yasukawa H, Kai M, Seki Y, Kuwahara F, *et*

al. Peripheral blood levels of matrix metalloproteases-2 and -9 are elevated in patients with acute coronary syndromes. J Am Coll Cardiol 1998; 32: 368-72.

5 Inokubo Y, Hanada H, Ishizaka H, Fukushi T, Kamada T, Okumura K. Plasma levels of matrix metalloproteinase-9 and tissue inhibitor of metalloproteinase-1 are increased in the coronary circulation in patients with acute coronary syndrome. Am Heart J 2001; 141: 211-7.

6 Yan JC, Wu ZG, Li L, Zhong RQ, Kong XT. The clinical implications of increased expression of CD40L in patients with acute coronary syndromes. Chin Med J 2002; 115: 491-3.

7 Peng DQ, Zhao SP, Li YF, Li J, Zhou HN. Elevated soluble CD40 ligand is related to the endothelial adhesion molecules in patients with acute coronary syndrome. Clin Chim Acta 2002; 319: 19-26.

8 Phipps RP, Koumas L, Leung E, Reddy SY, Blieden T, Kaufman J. The CD40-CD40 ligand system: a potential therapeutic target in atherosclerosis. Curr Opin Investig Drugs 2001; 2: 773-7.

9 Ozmen J, Bobryshev YV, Lord RS. CD40 co-stimulatory molecule expression by dendritic cells in primary atherosclerotic lesions in carotid arteries and in stenotic saphenous vein coronary artery grafts. Cardiovasc Surg 2001; 9: 329-33.

10 Mach F, Schonbeck U, Sukhova GK, Atkinson E, Libby P. Reduction of atherosclerosis in mice by inhibition CD40 sinalling. Nature 1998; 394: 200-3.

11 Wilson RF, Holida MD, White CW. Quantitative angiographic morphology of coronary stenoses leading to myocardial infarction or unstable angina. Circulation 1986; 73: 286-93.

12 Divies MJ. Stability and instability: two faces of coronary atherosclerosis. Circulation 1996; 94: 2013-20.

13 Schroeder AP, Falk E. Vulnerable and dangerous coronary plaques. Atherosclerosis 1995; 118: S141-9.

14 Aukrust P, Muller F, Ueland Thor BS, Berget T, Aaser E, Brunsvig BS, *et al*. Enhanced levels of soluble and membrane-bound CD40 ligand in patients with unstable angina. Circulation 1999; 100: 614-20.