Increased expression of CD40 ligand and C-reactive protein in patients with restenosis after percutaneous coronary intervention

Jin-Chuan Yan[1†], Yi Liang[1], Gen-Shan Ma[2], Jian Zhu[2], Yi Feng[2], Dan Luo[2], Zong-Gui Wu[3], Xian-Tao Kong[3], Ren-Qian Zong[3] & Lin-Zen Zhan[3]

[†]Author for correspondence
[1]Department of Cardiology, Affiliated Hospital of JiangSu University, China
Tel.: + 86 511 502 6169; E-mail: yanjinchuan@hotmail.com
[2]Zhong Da Hospital, Southeast University, Nanjing 210009, China
[3]Chang Zheng Hospital, Second Military Medical University, Shanghai 20003, China

Background: Inflammation plays a pathogenic role in restenosis after percutaneous transluminal coronary angioplasty. Elevated levels of C-reactive protein are one of the strongest prognostic factors in atherosclerosis. Increasing evidence demonstrates that the CD40–CD40 ligand (L) interaction plays a crucial role in the pathogenesis of atherosclerosis and coronary artery disease. However, its role in the pathophysiology of restenosis is still unclear. **Methods:** A total of 150 patients with percutaneous coronary intervention were investigated. The expression of CD40 and CD40L on platelets was analyzed by indirect immonofluorescence flow cytometry and serum-soluble CD40L level and C-reactive protein was determined by commercially available enzyme-linked immunosorbent assay. Patients with restenosis within a 6-month follow-up were observed in 150 consecutive patients who underwent percutaneous coronary intervention. **Results:** Restenosis occurred in 38 patients (25.3%). Patients who developed restenosis showed higher levels of CD40–CD40L system compared with nonrestenotic patients before percutaneous coronary intervention. All restenotic patients demonstrated a significant increase of CD40 (66.8 ± 4.4 mean fluorescence intensity [MFI]) and CD40L (14.2 ± 1.7 MFI) coexpression on platelets as well as soluble CD40L (14.0 ± 1.6 ng/ml) compared with nonrestenotic patients and controls at 6-month follow-up ($p < 0.001$). Elevated sCD40L and CD40L were significantly correlated with serum C-reactive protein levels after percutaneous transluminal coronary angioplasty and with lumen loss at 6-month follow-up. **Conclusion:** Our results suggest that the CD40L system levels are associated with late restenosis after percutaneous coronary intervention. This would suggest that restenosis is, in part, an inflammatory disorder.

Restenosis after percutaneous coronary intervention (PCI) is a major clinical problem and its pathophysiology has not yet been elucidated. Recently, the role of inflammation on the pathophysiology of restenosis after PCI has been emphasized, and C-reactive protein (CRP), which is an indicator of inflammatory reactions, has been reported to be related to restenosis [1]. The prognostic value and change in CRP after percutaneous intervention in patients undergoing drug-eluting stent insertion has also been demonstrated [2]. However, to date, a few conventional risk factors have been shown to be reliable risk predictors for restenosis after PCI.

Recently, increasing evidence shows that the CD40–CD40 ligand (L) interaction plays a crucial role in the pathogenesis of atherosclerosis and coronary artery disease [3,4]. Furthermore, we previously found that patients with acute coronary syndrome (ACS) had higher serum concentrations of soluble CD40L (sCD40L) than healthy volunteers or those with stable angina, and an obvious correlation was also observed between sCD40L concentration and complex coronary stenoses [5,6]. Recently, an increase in the CD40–CD40L system has been observed in patients with hypercholesterolemia and the circulating sCD40L has strong independent prognostic value among ACS [7,8].

Accordingly, it is temping to hypothesize that the CD40–CD40L interaction may also contribute to the development of restenosis after PCI; although a few studies have addressed that sCD40L was related to restenosis of PCI [9,10]. Limited patient numbers and no systematic detection restricted the results. CD40–CD40L system interaction may influence the inflammatory reaction after vessel injury, which is ultimately responsible for luminal renarrowing after PCI in humans. Therefore, the present study was designed to investigate the possible role of the CD40–CD40L system in restenosis after PCI and assess the correlation with CRP as well as lumen loss in PCI patients.

Materials & methods

Reagents

The following antibodies were used:

- Mouse-antihuman CD40
- Mouse-antihuman CD40L

Keywords: CD40, CD40 ligand, percutaneous coronary intervention, restenosis

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Table 1. Characteristics of the study groups.

Variable	No restenosis (n = 112)	Restenosis (n = 38)
Age (years)	63.1 ± 9.4	64.2 ± 8.7
Sex (male/female)	72/40	24/14
Stable angina/ACS	37/75	12/26
Diabetes (n)	33	11
Hypertension (n)	57	20
Cigarette smoking (n)	30	10
Hypercholesterolemia (n)	28	9
Medication		
β-blocker (n)	92	32
Nitrates (n)	54	17
Aspirin (n)	112	38
Statin (n)	104	35
Clopidogrel (n)	111	38
Culprit vessel		
LAD	52	17
LCX	24	8
RCA	36	13

$p < 0.05$ between two groups.
ACS: Acute coronary syndrome; LAD: Left anterior descending; LCX: Left circumflex coronary; RCA: Right coronary artery.

- CD61–fluorescein isothiocyanate (FITC)-conjugated antibody
- sCD40L enzyme-linked immunosorbent assay (ELISA) kit was from Bender Medsystems (PharMingen)

Patients & controls

The trial enrolled 150 patients with stable angina or ACS and all patients were documented by coronary angiography to have substantial coronary artery disease, with stenosis of at least 70% of the coronary artery diameter at a culprit lesion that was suitable for angioplasty. We chose drug-eluting stents for patients with diabetes or a diameter of the target vessel of 3.0 mm or less or long lesions (>20 mm). Patients with infection, tumor, liver or kidney diseases were excluded. Informed written consent was obtained from each patient (Table 1).

Box 1. Continuous luminal loss equation.

$$\text{Late loss} = \frac{\text{Postintervention minimal lumen diameter} - \text{Follow-up minimal lumen diameter}}{\text{Vessel size}} \times 100\%$$

Angioplasty procedure & follow-up evaluation

PCI and quantitative coronary angiography were performed according to standard techniques, as described previously [11]. All patients took clopidogrel for at least 9 months and aspirin for long periods. Patients were readmitted for a follow-up coronary angiography 6 months after angioplasty. We excluded three patients who failed to show up for repeat angiography. Restenosis was defined as a recurrent lumen diameter stenosis of more than 50% at the follow-up angiograph. The continuous luminal loss was defined according to the equation shown in Box 1. The vessel size is the value of the reference diameter function at the minimal position of the obstruction.

Blood sampling protocol

Rich-plasma platelet was performed, by a method described previously [12]. Briefly, peripheral venous blood was drawn into blood collection tubes containing sodium citrate. Citrated blood samples were either centrifuged (200 g for 10 min at room temperature) 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 for 10 min at 4°C). The supernatant was stored at -80°C until analysis and thawed only once.

Detection of CD40 & CD40L on platelets by flow cytemetry

Platelet immunostaining was performed as previously described [13]. Fixed blood was diluted 1:100 with phosphate-buffered saline and incubated with the first antibody (30 min, 4°C). Platelets were then incubated with phycoerythrin (PE)-conjugated second antibody (30 min, 4°C) 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 more than 98% positive for CD61.

Enzyme immunoassays

Serum and plasma samples were frozen and thawed only once. Specific immunoassays for sCD40L (sCD40 detection limit, 95 pg/ml; Bender Medsystems) and high-sensitive CRP (Immulyte hs-CRP, Diagnostic Product Corp.)

Table 2. CD40 system expression measured at different times between restenotic and nonrestenotic patients.

Time	CD40 (MFI)	CD40L (MFI)	sCD40L (ng/ml)
Before PCI	60.2 ± 7.0 vs 54.0 ± 6.5	11.2 ± 2.8 vs 9.2 ± 1.8	11.2 ± 2.6 vs 9.4 ± 1.7
24 h	68.2 ± 5.2* vs 54.8 ± 6.0	14.9 ± 2.0* vs 10.0 ± 1.8	14.2 ± 2.1* vs 9.9 ± 1.9
1 week	75.3 ± 5.9* vs 56.3 ± 6.2	17.1 ± 1.7* vs 10.9 ± 1.7	15.1 ± 1.7* vs 10.8 ± 1.9
2 weeks	67.2 ± 4.7* vs 52.6 ± 6.4	15.6 ± 1.5* vs 9.3 ± 1.9	13.7 ± 1.8* vs 9.4 ± 2.0
1 month	66.5 ± 5.0* vs 52.5 ± 6.7	14.1 ± 1.9* vs 9.4 ± 1.6	13.5 ± 1.6* vs 9.3 ± 1.8
6 months	66.8 ± 4.4* vs 51.8 ± 6.2	14.2 ± 1.7* vs 9.1 ± 1.5	14.0 ± 1.6* vs 9.4 ± 1.6

<0.001 vs before PCI.

MFI: Mean fluorescence intensity; PCI: Percutaneous coronary intervention.

were used in triplicate, as described previously [9]. At our laboratory, the intra- and inter-assay coefficients of variation were less than 5%.

Statistical analysis

Statistical evaluation was performed with Graph pad software (Prism 3.0) and SAS 8.0 software. Data were expressed as mean plus or minus the standard deviation and statistically compared by repeated measures. Correlation was evaluated using regressive analysis. The Spearman two-way test was used to assess the relationship between two quantitative variables with non-normal distribution. The Pearson two-way test was used to assess the relationship between two quantitative variables with normal distributions. p-values less than 0.05 indicate statistical significance.

Results

Clinical follow-up results

At 6 months follow-up, no patients developed myocardial infarction or sudden death during the study. Restenosis occurred in 38 out of 150 PCI patients (25.3%).

CD40–CD40L system expression in patients

Patients who developed restenosis showed higher levels of CD40 (60.2 ± 7.0 vs 54.0 ± 6.5 MFI; p = 0.0001) and CD40L (11.2 ± 2.8 vs 9.2 ± 1.8 MFI; p = 0.0017) expression on platelets as well as sCD40L (11.1 ± 2.6 vs 9.4 ± 1.7 ng/ml; p = 0.0043) compared with nonrestenotic patients in the samples collected before PCI. Furthermore, the CD40–CD40L system was analyzed in all patients before and 24 h, 1 week, 2 weeks, 1 month and 6 months after PCI, as can be seen in **Table 2**. Enhanced CD40 system after PCI persisted as statistically significant throughout the study in the restenotic patients, whereas they normalized 2 weeks after PCI in the nonrestenotic patients (**Table 2**).

CRP levels & CD40 system: relationship in patients

Patients who developed restenosis showed significantly higher CRP levels (1.45 ± 0.35 vs 0.93 ± 0.19 ng/ml; p = 0.0001) than nonrestenotic patients in this study. Moreover, CRP remained high in the follow-up restenotic patient group. Accordingly, a positive correlation was found between sCD40L, CD40L expression and CRP at each collection time in the restenotic patient group (**Table 3**). By contrast, we found no evidence for an association beteween CRP, sCD40L and CD40L in nonrestenotic patients. No correlation was also found between CD40 expression and CRP in all patients.

CD40 system & PCI late lumen loss

To investigate whether the CD40 system may contribute to luminal restenosis after PCI, we assessed the association between the CD40 system expression and the degree of luminal renarrowing at 6 months after PCI. Lumen loss showed a significant positive correlation with sCD40L and CD40L at 6 months. However, a significant correlation of luminal loss with expression of CD40 on platelets was not found in patients ($r^2 = 0.02513$; p = 0.3418; n = 38).

Discussion

CD40–CD40L interaction is involved in the inflammatory pathogenesis of atherosclerosis, although clinical data about its role in stent restenosis are still limited. Restenosis is a serious complication after PCI. PCI leads to activation of platelets and white cells and triggers an acute inflammatory response that plays a major role in the pathogenesis of complications after this procedure [14,15]. Increasing evidence now supports the role of inflammation in restenosis after PCI [16]. Recently, Cipollone and colleagues reported that preprocedural levels of soluble CD40L were

Table 3. The relationship between the CD40 system and CRP levels in restenotic and nonrestenotic patients.

	CD40		CD40L		rCD40L	
	Res	***No Res***	***Res***	***No Res***	***Res***	***No Res***
R2 (0)	0.1009	0.1135	0.3813*	0.0346	0.3958*	0.0372
R2 (1)	0.0140	0.0046	0.4851*	0.0450	0.5853*	0.0347
R2 (7)	0.0451	0.0342	0.4701*	0.0613	0.4792*	0.0523
R2 (14)	0.0849	0.0540	0.4211*	0.1035	0.3004*	0.0393
R2 (30)	0.0965	0.0093	0.5142*	0.0153	0.3749*	0.0361
R2 (180)	0.0691	0.0430	0.5896*	0.0885	0.5002*	0.0597

*$p < 0.0001$

associated with late restenosis after percutanerous transluminal coronary angioplasty (PTCA) [17]. In this study, we investigated both the CD40–CD40L system (including CD40, CD40L expression on platelets and circulating soluble CD40L), CRP levels and lumen renarrowing and the relationship between them. We found that the restenotic patient group had an increased level of CD40 system and CRP. PCI resulted in significant increases in CD40, CD40L, rCD40L and CRP in a short time for all patients. However, patients who developed restenosis showed sustained higher levels of CD40L, rCD40L and CRP than nonrestenotic patients.

Studies on the cellular distribution of CD40L indicate that more than 95% of circulating sCD40L exists in platelets [18,19]. Notably, we recently demonstrated that the increased circulating rCD40L was from activated platelets in hypertension patients [20]. In fact, PCI is known to disrupt the endothelium, resulting in the exposure of thrombogenic surfaces that support the adhesion, activation and aggregation of platelets. Thus, the platelet-rich thrombi may be the major intravascular source of enhanced expression of CD40 system both on the platelets surface and in the circulating environment as they shed sCD40L.

CRP has consistently been shown to have a close correlation with restenosis or major cardiovascular events [21–23]. Most studies have focused on the importance of elevated hs CRP levels before PCI and immediately after. However, our study showed a significantly sustained high level of high sensitivity (hs)CRP in the restenotic patients group. This result indicates patients with long-term enhanced levels of CRP have a higher incidence of restenosis.

Recently, considerable evidence implicates that the CD40–CD40L interaction plays a crucial role in multiple stages of atherosclerosis. Therefore, we suspected the CD40 system was also associated with the restenosis after PCI. In this study, we demonstrated that CD40L expression was not only significantly correlated with CRP levels but also with the luminal loss in restenotic patients. This result may indicate an association between CD40L changes and the occurrence of restenosis. This new finding may also indicate a reliable risk predictor for restenosis and provide a potential preventive strategy for restenosis, such as using an anti-CD40L antibody to prevent lumen renarrowing after PCI. Our results were slightly different from those reported by Yip [10]. This may be due to the numbers of patients and the detection time. Further detailed studies should be performed.

A limitation of our study is the possibility that the small cohort of patients and the limited sample collection time may have affected the results. Therefore, further large-scale studies and prolonged follow-up should be performed to illustrate that the clinical value of the CD40L level, independently or in combination with other markers, is a risk factor for restenosis. Simultaneous assessment of the CD40 system and other factors yields independent and complementary prognostic information, thus enabling more powerful prediction of restenosis.

Executive summary

- Increased CD40 system expression was associated with late restenosis after percutaneous coronary intervention (PCI).
- Restenosis is in part an inflammatory disorder.
- C-reactive protein (CRP) levels remain considerably high in the follow-up restenotic patients group and a positive correlation was found between CD40 ligand (L) expression and CRP.
- Lumen loss was significantly positively correlated with CD40L levels at 6 months after PCI.

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