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REVIEW

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CD40/CD40L system and vascular disease

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Abstract Several distinct lines of investigation in the context of atherosclerosis dealing with low-grade inflammation, oxidative stress and platelet activation are now emerging, with CD40/CD40L system as the missing link. CD40 ligand is a transmembrane glycoprotein structurally related to tumour necrosis factor-α and more than 95% of the circulating CD40L derives from platelets. CD40L appears as a multiplayer of several cell types in the inflammatory network. The peculiarity of CD40L as an inflammatory mediator derived from platelets expands the functional repertoire of platelets from players of haemostasis and thrombosis to powerful amplifiers of inflammation by promoting the release of cytokines and chemokines, cell activation and cell–cell interactions. The multifunctional role of CD40L, as a simultaneous activator of all these systems, further blurs the intricate relationship between such events both in the physiological systems and the pathological derangement occurring in atherothrombosis.

Keywords sCD40L · Inflammation · Atherosclerosis · Thrombosis

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Introduction

CD40L is gaining much attention for its role in the initiation and progression of atherosclerosis. Several distinct lines of investigation in the context of atherosclerosis dealing with low-grade inflammation, oxidative stress and platelet activation are now emerging, with CD40/CD40L system as the missing link. CD40 ligand (CD40L, CD154) is a 39-kD transmembrane glycoprotein structurally related to tumour necrosis factor-α (TNF-α), originally thought to be restricted to stimulated CD4-positive T cells [1, 2], mast cells and basophils [3]. Subsequent studies demonstrated its expression on platelets, carrying preformed CD40L, which is rapidly translocated onto the cell surface following activation [4]. The receptor for CD40L, CD40, is constitutively expressed on monocyte/macrophages, endothelial cells (ECs), smooth muscle cells (SMCs) [5] and platelets [6]. Studies on the cellular distribution of CD40L indicate that more than 95% of the circulating CD40L derives from platelets [7]. Henn and colleagues have shown that CD40L, cryptic in unstimulated platelets, is expressed on the surface of platelets within seconds of platelet activation and then cleaved to generate a soluble, trimeric fragment, sCD40L [4]. Multiple platelet agonists, including collagen, thrombin and ADP, are able to induce the exposure of sCD40L from platelets [4]. The translocation of sCD40L on the platelet surface requires a few seconds or minutes, similar to the expression of P-selectin, whereas the shedding of the molecule into the circulation is a slower event, lasting 30–45 min depending on the agonist inducing activation [8]. It is now generally accepted that sCD40L is generated by proteolytic cleavage mediated through activation of a membrane-bound protease, possibly a metalloproteinase (MMP), although the involvement of intracellular structures and reactions has been also proposed, i.e., actin polymerisation [9, 10]. In addition, sCD40L may be possibly involved in a self-perpetuating feedback loop, whereby it binds platelet-bound

CD40 leading to further proteolysis of membrane-bound CD40L, with consequent generation of further sCD40L [6].

There are conflicting data as to whether sCD40L stimulates resting platelets by binding to constitutively expressed CD40 during direct cell–cell contact, thus eliciting proinflammatory responses. Inwald et al. demonstrated granule release and enhanced P-selectin expression after incubation of platelets with trimeric sCD40L [11], suggesting that the biological activity of sCD40L may depend on its existence in a biologically active trimeric structure (as in the case of membrane-bound CD40L) [12]. The prothrombotic activity of sCD40L may be attributable to its KGD peptide sequence as infusion of sCD40L with an altered KGD sequence did not reverse the normal phenotype in CD40L-deficient mice [7]. This sequence in turn allows its binding to glycoprotein IIb/IIIa, with consequent stabilisation of arterial thrombi [7]. Proof that sCD40L is a GPIIb/IIIa ligand was obtained through experiments of direct binding of sCD40L to purified glycoprotein IIb/IIIa [7]. More recently, *in vitro* studies showed that GPIIb/IIIa antagonists (eptifibatide, abciximab and tirofiban) are capable of inhibiting the release of sCD40L in a dose-dependent manner [8, 10, 13], although translocation of CD40L from intraplatelet stores to the surface was unmodified [10]. Taken together, these observations strongly implicate CD40L in the triggering and perpetuation of platelet activation.

CD40/CD40L interactions have also been involved in inflammation and thrombosis. In fact, CD40 and CD40L are coexpressed by virtually all of the cells involved in the processes of atherosclerosis at all stages, such as vascular endothelial cells, smooth muscle cells, macrophages, activated T lymphocytes and platelets [14]. CD40/CD40L interaction on these cellular types triggers a series of events occurring in the vascular wall and in the circulation during the ongoing inflammatory response, events which taken together identify the inflammatory and prothrombotic phenotype observed in both the early and late stages of atherosclerosis. Ligation of CD40 on ECS and VSMCs induces the expression of adhesion molecules such as vascular cell adhesion molecule-1 (VCAM-1), intercellular cell adhesion molecule-1 (ICAM-1) and P-selectin, which in turn promote the recruitment and extravasation of monocytes and lymphocytes at the site of vascular injury [14]. Further recruitment of lymphocytes is elicited by CD40L-induced secretion of cytokines such as interleukin-1 (IL-1), interleukin-6 (IL-6) and tumour necrosis factor-α (TNF-α) by several cells, thus fostering a predominantly Th1 cytokine-driven immune reaction, characteristic for atherogenesis, and of chemokines, such as macrophage inflammatory protein-1α (MIP-1α), MIP-1β regulated upon activation normal T-cell expressed and secreted (RANTES), stromal derived factor-1 (SDF-1) and monocyte chemoattactant protein-1 (MCP-1) [5]. CD40/CD40L signalling in endothelial cells results in the production of reactive oxygen species (ROS), which antagonise endothelial NO production thus promoting endothelial dysfunction. In addition, this signalling also results in upregulation of the interstitial collagenases MMP-1, MMP-8 and MMP-13, contributing to impairment of plaque stability, and in inhibition of endothelial cell migration, thus preventing reendothelialisation of plaque erosion. Finally, CD40 signalling induces tissue factor (TF) expression [15–17], which, while promoting blood coagulation, is also able to activate platelets, which in turn enhance further CD40L shedding with consequent amplification of the inflammatory reaction. In addition, sCD40L increases stimulation-induced platelet release of ROS through activation of Akt and p38 MAP kinase signalling pathways [18]. Recent *in vitro* and *in vivo* experiments in a mouse model elucidated a novel alternative pathway for CD40L-mediated inflammation, by interaction with the monocyte/macrophage integrin MAC-1. This interaction enhances adhesion and migration of inflammatory cells and myeloperoxidase release *in vitro*, and inhibition of MAC-1 in low-density lipoprotein (LDL) receptor-deficient mice attenuates lesion development and decreases macrophages accumulation [19].

All these events may potentially induce or facilitate an acute thrombotic event (Fig. 1).

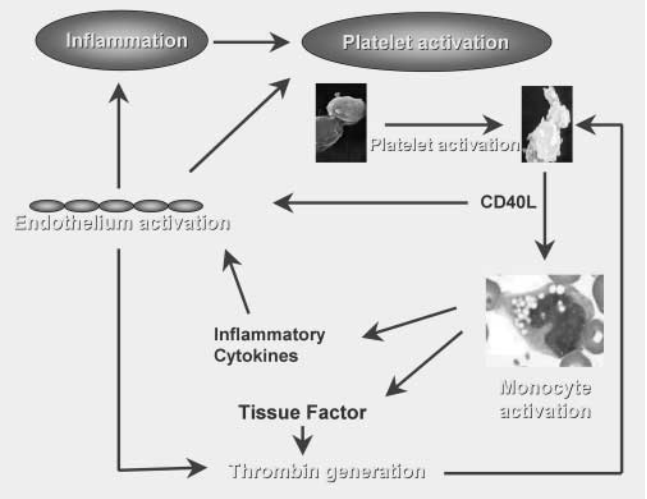

Fig. 1 Role of CD40L in the complex interplay between inflammation, endothelial activation/dysfunction and platelet/coagulative activation. Soluble CD40L, shed from platelets upon platelet activation, triggers monocyte activation, leading to release of inflammatory cytokines and TF expression with thrombin generation, and activation of the endothelium, resulting in further inflammation and platelet activation

CD40L and vascular disease

Coronary artery disease

The pivotal role of platelet activation in atherothrombosis, coupled with the finding that most of sCD40L is derived from platelets, has made CD40L an interesting subject in the setting of cardiovascular disease and of acute coronary syndrome (ACS).

Clinically relevant concentrations of human sCD40L increased the expression of its receptor CD40 in human coronary artery endothelial cells through a mechanism mediated by oxidative stress and extracellular signal-regulated kinase (ERK) 1/2 activation, suggesting a mechanism of amplification of CD40L biological function contributing to endothelial dysfunction and platelet activation in the coronary endothelium [20].

Elevated levels of sCD40L have been reported in patients with both stable or unstable angina (UA) [21], acute myocardial infarction (AMI) [22] and diabetes with angiographically proven coronary artery disease (CAD) [23]. Aukrust and colleagues also performed a series of experiments *ex vivo* to test the hypothesis that the CD40/CD40L dyad may play a pathogenetic role in both the long-term atherosclerotic process and in the triggering of ACS [21]. They also addressed the important issue of the cellular origin of the enhanced sCD40L. In fact, they showed that platelets are able to release large amounts of sCD40L *ex vivo* when stimulated with the thrombin receptor-antagonist peptide SFLLRN in patients and controls. Moreover, increased surface expression of CD40L on T lymphocytes and increased *in vitro* shedding of sCD40L from these cells has also been reported in patients with UA [21]. Thus, T lymphocytes and particularly platelets cause the major contributor to increased circulating sCD40L in the setting of UA. Interestingly, sCD40L-rich serum from patients with UA was able to induce enhanced release of MCP-1 from monocytes, giving rise to the hypothesis that sCD40L may be a contributor rather than the consequence of UA, being responsible for the progressive plaque instability and eventually plaque rupture. On the other hand, procedures such as cardiopulmonary by-pass [24] and percutaneous transluminal coronary angioplasty (PTCA) [25] have been shown to induce sCD40L release, suggesting that elevated sCD40L could be a mere consequence of plaque rupture. To recompose these apparently opposite findings in a unifying hypothesis, it has been speculated that repeated episodes of "minor plaque ruptures" may occur before the onset of clinically manifest coronary event, inducing further elevation of sCD40L, and such a vicious circle may be operating in these patients and may contribute to the progression of the disease.

Some additional information about this issue emerges from the study by Cipollone and coworkers [25] and subsequent studies [26], which demonstrated that preprocedural levels of sCD40L are predictive of enhanced inflammatory response and restenosis after PTCA. A mechanistic basis for this clinical study is provided by the work by Urbich et al., who showed that CD40L inhibits endothelial cell migration by increasing production of ROS [27], thus providing a mechanism by which sCD40L generated by thrombosis could promote restenosis.

A prospective study demonstrated that raised sCD40L is a risk factor for future cardiovascular events in healthy women [5]. Heeschen and coworkers further established the predictive value of this marker by evaluating the clinical relevance of sCD40L in patients presenting with chest pain [22]. In 1088 patients from the c7E3 Fab Antiplatelet Therapy in Unstable Refractory Angina (CAPTURE) Study group, increased sCD40L among patients with chest pain undergoing PTCA identified subjects at risk of death and non-fatal AMI. The increased risk associated with elevated sCD40L was reduced with abciximab pretreatment, a potent platelet glycoprotein IIb/IIIa receptor antagonist. In contrast to troponins, the predictive value of sCD40L levels with respect to the benefit of abciximab is independent of the presence of recent AMI. As a matter of fact, sCD40L identifies the subgroup of patients at highest risk for cardiac events even among those negative for troponin. Whereas positivity of troponins in patients with ACS indicates myocardial necrosis, sCD40L levels reflect the inflammatory thrombotic activity of the culprit lesion in recruiting activating platelets. As the outcome of ACS is highly dependent on the interplay between inflammation and thrombosis, an approach including measurements of these two processes in addition to markers of necrosis may improve our understanding of the underlying pathophysiological processes and allow better assessment of plaque instability.

A subsequent study [28] evaluating plasma sCD40L levels in patients with ACS who eventually reached the study endpoint (death/AMI/congestive heart failure) and in patients who did not, showed significantly higher levels of sCD40L in cases than controls. Patients with the highest sCD40L levels were significantly more likely to develop an AMI or the composite endpoint of death/AMI compared to the patients in the lowest quartiles. Hazard ratios adjusted for troponin I and CRP showed that sCD40L is an independent predictor of the risk of death or MI at 10 months after an ACS.

Recently, genetic polymorphism C807T of platelet glycoprotein Ia has been shown to increase the risk for premature MI and is an independent predictor of sCD40L levels during the acute phase of premature MI and one year after the event [29].

Moreover, in patients with non-ST elevation MI a single nucleotide polymorphism (-3459 A>G) in the CD40L gene is a novel regulator of sCD40L concentrations, and increased sCD40L are predictive of MI and of the efficacy of antithrombotic treatment with dalteparin [30].

These studies do not have the power to establish the causal role of CD40L in the pathogenesis of ACS, but these findings raise the question whether elevated sCD40L levels are a reflection of the pathogenetic role of CD40L in the inflammatory and thrombotic processes or whether they are a mere consequence of the clinical event, namely the result of platelet release after thrombus formation. Even in the latter case of sCD40L elevation as an epiphenomenon, it may constitute a reliable marker of platelet activation and of unstable, prone to rupture plaque. Given

the above findings, it would be worthwhile to evaluate whether lowering the levels of this molecule translates into an improvement of cardiovascular outcomes.

Cerebrovascular disease

Elevated plasma levels of sCD40L were detected in patients undergoing high-resolution magnetic resonance imaging of carotid atheroma with evidence of intraplaque lipid pool [31].

Our group has recently evaluated 42 patients with asymptomatic low-grade carotid stenosis and at least one cardiovascular risk factor [32]. Patients had higher sCD40L as well as C reactive protein (CRP) and IL-6 than controls. Subjects were reviewed annually for a median follow-up of 8 years and 14 patients experienced a cardiovascular event. Cox regression analysis showed that only high sCD40L plasma levels predicted cardiovascular risk, independently of cardiovascular risk factors. Thus, downregulation of this system may represent a potential therapeutic target capable of inducing a more stable carotid plaque phenotype.

Both the ligand and its receptor are overexpressed in human and experimental atherosclerotic plaques, in particular in rupture-prone or ruptured plaques [14].

The CD40L expression on platelets from patients with ischaemic stroke was higher than on platelets from patients with asymptomatic carotid stenosis and from normal subjects [33]. Garlichs and colleagues [34] show CD40/CD40L upregulation in patients with acute cerebral ischaemia. Patients exhibited a significant increase of platelet and T-cell expression of CD40L, as well as of CD40 on monocytes, as compared to controls. Interestingly, CD40L upregulation persisted after 3 months since the initial event. In this light it would be interesting to examine whether sCD40L in the subacute phase may predict the recurrence of ischaemic events [35].

Recently, enhanced sCD40L levels were described in patients with Alzheimer's disease, suggesting a role for CD40L in the pathogenesis of this disease [36]. It is possible to hypothesise that increasing CD40L is a surrogate indicator for the switch from a more stable collagen-rich phenotype to an unstable, rupture-prone phenotype [37], which is ultimately responsible for an acute cerebral ischaemic event or for its recurrence. Whether inhibition of CD40/CD40L dyad may translate into a reversal of such a phenotypic switch remains to be elucidated through intervention studies.

Peripheral artery disease

Recently, the hypothesis of a role for sCD40L in the setting of peripheral artery disease was tested both cross-sectionally and with an intervention study [38]. Plasma sCD40L, in addition to other platelet indices such as soluble P selectin, platelet microparticles and platelet surface expression of CD62 and CD63, is raised in peripheral atherosclerosis and is increased after peripheral artery angioplasty, although levels seem unrelated to clinical severity. sCD40L levels did not correlate with other markers, suggesting that platelets may not be the unique source of sCD40L, and that other cells may contribute to plasma levels.

Pulmonary arterial hypertension

Pulmonary arterial hypertension (PAH) is a complex disease that recognises both inflammation and platelet activation in its pathophysiology [39]. Damas and coworkers have recently reported an upregulation of the CD40/CD40L system even in this setting [40]. Patients with primary and secondary PAH but not those with chronic thromboembolic pulmonary hypertension had increased plasma sCD40L compared to controls. In addition, in the patient group as a whole, a positive correlation was found between sCD40L and prothrombin F1+2, suggesting CD40L and platelet activation associated with the ongoing thrombus formation in this setting. Prostacyclin therapy for 3 months did not affect sCD40L levels, despite clinical benefit. This study suggests that CD40L may play a role in the pathogenesis of PAH, possibly triggering a complex chain of events played by platelets and ECs through chemokine-related mechanisms.

Heart failure

Stumpf et al. [41] have recently shown enhanced expression of CD40L on the platelet surface during chronic heart failure (HF), further supporting enhanced platelet activation in this disorder.

Serum levels of sCD40L were measured in 236 patients with acute HF following MI treated with either angiotensin-converting enzyme (ACE) inhibition or angiotensin II blockade and followed for 2 years, and in 116 patients with chronic HF [42]. Patients with acute HF had increased sCD40L levels, particularly those with severe HF, diabetes or hypertension; when these patients were followed longitudinally, persistently raised sCD40L levels were found throughout the observation period with no effect of captopril or losartan; the increase in sCD40L during follow-up was not seen in patients receiving warfarin therapy; patients with chronic HF also had raised sCD40L, significantly correlated with clinical severity, neurohormonal dysregulation and left ventricular dysfunction; studies from different blood compartments suggest that the vasculature of lower extremities and the failing myocardium itself may produce and secrete sCD40L in chronic HF.

The correlation between sCD40L and LV dysfunction suggests that increased sCD40L levels in HF may not only be a parameter of platelet activation but could also reflect other pathogenic mechanisms in myocardial failure, including activation of MMPs and induction of inflammatory cytokines and chemokines. Whatever the mechanisms, the CD40-expressing cardiomyocytes may directly interact with CD40L, either in its soluble form or expressed on the surface of infiltrating T cells and platelets, contributing to persistent tissue inflammation and remodelling within the failing myocardium. These findings suggesting enhanced release of sCD40L within the coronary circulation further support enhanced CD40/CD40L interaction within the myocardium.

CD40L and cardiovascular risk factors

Diabetes mellitus

The strong correlation between diabetes mellitus (DM) and atherosclerosis suggests that both conditions may share a common background [43]. A clustering of variables related to chronic low-grade inflammation, oxidative stress and platelet activation is emerging as a conceivable "common soil" that may influence the development of both diseases [44].

In vivo platelet activation has been reported previously in diabetes [45, 46] and increased plasma levels of CD40L have been described recently in both type 1 (T1) and type 2 (T2) DM [23, 47]. Moreover, significantly increased co-expression of CD40 and CD40L on platelets of diabetic patients compared with nondiabetic controls has been reported, with a significant correlation of sCD40L with CD40L expression on platelets [48]. Surface expression of collagen receptor Fc receptor-gamma/glycoprotein VI is enhanced on platelets in T2DM and mediates release of CD40L and activation of endothelial cells, thus suggesting enhanced collagen-mediated platelet activation in diabetes contributing to thromboischaemic complications [49].

In T1DM, enhanced sCD40L levels have been detected in patients both with [50] or without micro- and macrovascular complications [51]. Serum CD40L is an independent determinant of intima-media thickness of carotid artery in young Japanese T1DM patients [52]. Patients with T1DM without other cardiovascular risk factors show increased CD40L expression and platelet-monocyte aggregation [53], possibly contributing to the prothrombotic and proinflammatory milieu found in diabetes. In addiction, increased sCD40L levels in patients with T1DM and microangiopathy have been associated with platelet hyperactivity, as assessed by platelet P-selectin expression and soluble P-selectin [50].

The highly significant correlation between plasma CD40L levels and the urinary excretion rate of 11-dehydro-TXB_2, a non-invasive index of *in vivo* platelet activation, supports the likelihood of CD40L release during TXA_2-dependent platelet activation in T2DM [54]. This relation is in keeping with previously reported evidence that CD40L is rapidly upregulated during platelet activation [4] and that platelet CD40 itself provides a mechanism for platelet activation [11]. Diabetics showed significantly reduced plasma CD40L after 7 days of aspirin. In addition, improved metabolic control after a 4-week intensive diabetes programme led to reduction in both sCD40L and 11-dehydro-TXB_2 [54]. The observation that both improved metabolic control and low-dose aspirin, two independent interventions down-regulating platelet activation in this setting, significantly reduced plasma CD40L levels, without any measurable impact on systemic inflammation, as reflected by the non-significant change in CRP plasma levels, strengthens the hypothesis of a contribution of platelets to enhanced CD40L release in T2DM [54]. In addition, the positive correlation observed between enhanced sCD40L and 8-iso$PGF_{2\alpha}$ is in line with the report of increased production of endothelial ROS by CD40L [27], suggesting that in T2DM the release of sCD40L from activated platelets may contribute to increased oxidant stress. Increased lipid peroxidation and persistent platelet activation have previously been reported in patients with T2DM [45, 46]. Thus, on the basis of these findings, a possible vicious cycle may be suggested in which inflammatory stimuli involving CD40L upregulation induce increased lipid peroxidation with consequent platelet activation resulting in further oxidant stress [4] (Fig. 2).

Recently, Lim et al. reported a strong correlation between sCD40L and both IL-6 and TF, supporting a link between the CD40/CD40L system and hypercoagulable

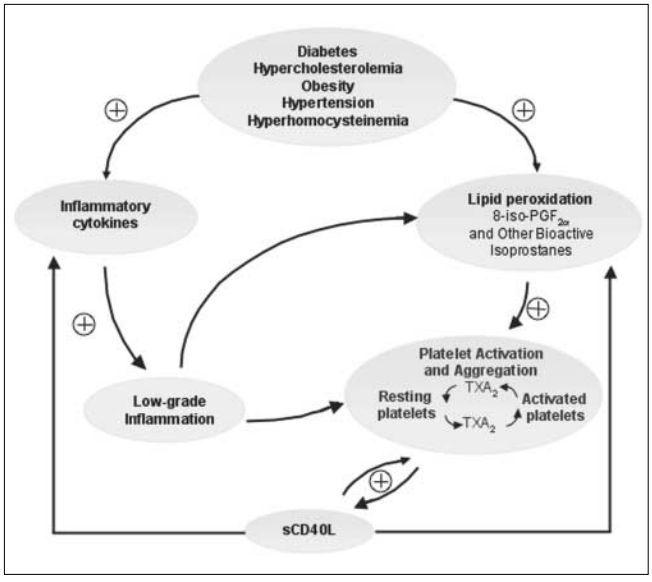

Fig. 2 Role of CD40L in the biochemical mechanisms linking cardiovascular risk factors, inflammation, lipid peroxidation and platelet activation. Potential amplification loops sustaining this chain of events are also shown. *PG*, prostaglandin; *sCD40L*, soluble CD40 ligand; *TX*, thromboxane

state in diabetes [55]. Moreover, elevated plasma sCD40L in patients with diabetes can be reduced by a 1-year multifactorial cardiovascular risk intervention strategy, consisting of ACE inhibitor-based blood pressure control, statin-based lipid lowering, sulphonylurea-based glycaemic control and antiplatelet therapy [55].

The molecular mechanisms linking sCD40L to the accelerated atherosclerosis in diabetes are not yet completely identified. To test the hypothesis that sCD40L is involved in the process of vascular complications in diabetes by triggering a complex group of inflammatory reactions both in vascular endothelial cells and in circulating monocytes/macrophages, we provided evidence in humans that enhanced sCD40L in both T1DM and T2DM is directly responsible for endothelial dysfunction and monocyte activation, demonstrating a positive correlation between sCD40L and ICAM-1, VCAM-1, E-selectin and MCP-1 in diabetic patients [56].

Finally, advanced glycation end-products have been identified as potential triggers of expression and release of sCD40L in diabetic patients [57].

Hypercholesterolaemia

There are several lines of evidence that implicate CD40/CD40L signalling in the vascular pathology associated with hypercholesterolaemia. High cholesterol levels have been associated with enhanced thrombotic risk, possibly related to enhanced persistent platelet activation [58] and thrombin formation [59] observed in this setting. sCD40L is related to cholesterol metabolism as assessed by cholesterol synthesis pathways in patients with moderate hypercholesterolaemia [60]. Patients with moderate hypercholesterolaemia exhibit increased *ex vivo* expression of CD40L and P-selectin on platelets and elevated CD40 expression on monocytes [61]. A larger study [62] showed that in patients with hypercholesterolaemia, increased plasma sCD40L levels have been positively associated with *in vivo* platelet activation, as reflected by plasma P-selectin and urinary 11-dehydro-thromboxane B_2, and with procoagulant state, as reflected by FVIIa and F1+2. Thus, it appears that the CD40/CD40L dyad may provide the mechanistic framework linking inflammation and prothrombotic state in this setting.

A further study was performed to test the hypothesis that CD40L-induced prothrombotic state is mediated by enhancing oxidative stress in the setting of hypercholesterolaemia [63]. Plasma sCD40L, 8-hydroxy-2'-deoxyguanosine, a marker of oxidative stress, and F1+2 levels were significantly higher in patients with hypercholesterolaemia as compared with controls. An additional *in vitro* study showed that CD40L overexpresses TF and increases the thrombin generation rate by an oxidative stress-mediated mechanism that requires activation of NADPH oxidase [64]. This mechanism, in turn, may be responsible for both the accumulation of oxidised LDL within the macrophages, and the prothrombotic state within the plaque. A short-term (3 days) treatment with atorvastatin was able to significantly reduce platelet CD40L and thrombin generation, independently of the lipid-lowering effect of the statin [64].

Cigarette smoking

Cigarette smokers show significantly increased sCD40L levels, surface expression of CD40L on platelets and T cells and of CD40 on monocytes and increased platelet-monocyte aggregates when compared to age- and gender-matched non-smokers [65]. In addition, the surface expression of CD40 on monocytes and of CD40L on platelets correlates with recent intake of nicotine, as assessed by plasma cotinine concentrations and by the number of cigarettes smoked. This finding may represent one of the possible explanations for the increased cardiovascular risk associated with cigarette smoking.

Obesity

We have reported that android obesity is associated with enhanced lipid peroxidation and persistent platelet activation, both improvable by successful weight loss [66]. These abnormalities are driven by inflammatory triggers related to the degree of abdominal adiposity. We found significantly higher plasma CRP levels, in association with enhanced oxidative stress and platelet activation in otherwise healthy women with visceral obesity [66]. Other authors showed increased CD40L levels in patients with severe obesity [67] and that weight loss over a 16-week period of caloric restriction was associated with significant reductions in sCD40L and oxidative stress [68]. In a further study by our group, CD40L plasma levels were elevated in individuals characterised by insulin resistance; however, its relation to increased platelet activation seems to be largely explained by differences in waist-to-hip ratio (WHR) and insulin sensitivity. Successful weight loss over a 12-week period was associated with a statistically significant increase in S_I and decreases in CD40L, CRP and in urinary 11-dehydro-TXB_2 excretion [69].

Arterial hypertension

Recently, 150 patients with different degree of arterial hypertension were evaluated for the expression of the CD40 system. All patients showed a significant increase of CD40 and CD40L coexpression on platelets as well as sCD40L levels compared with controls, with a slight correlation with blood pressure, suggesting that arterial hypertension is in part an inflammatory disorder [70].

Moreover, nondipper hypertensive patients have enhanced plasma CD40L levels as compared to dippers, with CD40L as the main determinant of IMT [71].

In essential hypertensive patients, microalbuminuria, a recognised marker of preclinical atherosclerosis, is not accompanied by enhanced plasma CD40L concentrations in comparison to hypertensive patients with normoalbuminuria [72].

Metabolic syndrome

Metabolic syndrome is gaining recognition as a multiplex cardiovascular risk factor. The presence of metabolic syndrome is independently associated with elevated sCD40L, CRP and coronary disease severity in CAD patients requiring interventional treatment of stable angina [73]. Patients with metabolic syndrome without cardiovascular disease had significantly higher sCD40L and sP-selectin compared with subjects without metabolic syndrome [74]. Moreover, increased sCD40L levels are related to enhanced thrombotic tendency, as shown by the relationship with prothrombin fragment F 1+2 [75].

Therapeutic implications

Thiazolidinediones

Thiazolidinediones, novel insulin-sensitising antidiabetic agents, act as peroxisome proliferator-activated receptor-γ (PPAR-γ) agonists and have been shown to exhibit antinflammatory and antiatherogenic properties [76, 77]. Treatment of diabetic subjects with a thiazolidinedione-type drug results in decreased sCD40L levels [23, 47].

A recent study [78] showed that human bone marrow megakaryocytes and platelets express PPAR-γ, and rosiglitazone is able to prevent thrombin-induced CD40L surface expression and release of CD40L and TXB_2. These results suggest that the reduced plasma levels of CD40L could derive from inhibition of platelet release of CD40L by the dampening effects of the PPAR-γ agonist drug. In this light it is conceivable to think that the antiinflammatory effects of thiazolidinediones may be mediated by modulation of platelet activation, thus identifying in the platelet a new target cell for this class of drugs.

Lipid-lowering drugs

In addition to the well known lipid-lowering effects, statins have been shown to exert anti-inflammatory and antiatherogenic properties [79, 80]. To better address this issue, several authors have investigated the effects of these drugs on inflammatory markers, including CD40L. Atorvastatin, cerivastatin and simvastatin were able to decrease in a dose-dependent manner the constitutive as well as oxidised LDL- or cytokine-induced expression of the receptor/ligand dyad in human vascular endothelial cells, smooth muscle cells and macrophages [81]. Moreover, statins have been shown to decrease CD40 expression and CD40-related activation of vascular cells, effects partially reversed by the HMG-CoA reductase product mevalonate [82, 83]. Both simvastatin and fenofibrate markedly reduced plasma levels of CD40L, as well as CRP, IL-1β, and improved endothelium-dependent vascular reactivity in patients with combined hyperlipidaemia [84]. In a substudy in the Atorvastatin versus Simvastatin on Atherosclerosis Progression (ASAP) trial, sCD40L levels were found to be about 27 times higher in 110 asymptomatic patients with familial hypercholesterolaemia as compared to controls [85]; statin therapy over 2 years markedly downregulated sCD40L levels, regardless of the statin used and with no correlation with the degree of cholesterol lowering. Recently, simvastatin, losartan and combined therapy significantly reduced sCD40L in hypercholesterolaemic hypertensive patients, to the greatest extent in subjects with high baseline CD40L levels [86].

In addition to their role in modulating CD40L in the setting of asymptomatic hypercholesterolaemia [81, 87], statins have proven effective in patients with stable CAD [88] and ACS [89, 90]. In patients with ACS enrolled in the Myocardial Ischemia Reduction with Aggressive Cholesterol Lowering (MIRACL) study [91], atorvastatin started within 24–96 h of admission abrogated the risk of recurrent cardiovascular events associated with high sCD40L over 16 weeks of treatment. Interestingly, atorvastatin did not significantly affect sCD40L concentrations over the treatment period. To explain this paradox, the authors speculate that the target of statins also involves downstream events in the CD40L pathway and signalling; in addition, it involves other inflammatory and thrombotic events marked by elevated sCD40L [91]. Given the detrimental effects of CD40L in the pathogenesis and progression of atherothrombosis, the ability of statins to lower the expression of this molecule may have potential therapeutic implications.

Antioxidants

As stated above, several lines of evidence substantiate a role of oxidative stress in enhancing CD40L expression [81, 92]. In particular CD40L downregulation in the setting of a deficit in gp 91 phox, the central care of NADPH oxidase, unravelled a role for O_2 generated by NADPH oxidase as a trigger of CD40L expression [92]. These interest-

ing data paved the way to studies testing the efficacy of antioxidants as modulators of CD40L release by platelets.

Recently, the adjunct of vitamin C *in vitro* to platelets stimulated with collagen, dose dependently inhibited platelet CD40L expression with no effect on platelet aggregation, and vitamin C infusion (1 g per 45 min) to healthy volunteers was associated with a significant and concurrent decrease of platelet O_2^- and CD40L by approximately 70% [93], independently of agonist-induced platelet aggregation.

In addition, CD40L exerts some of its detrimental effects by enhancing release of ROS in platelets [18] and endothelial cells [27], suggesting an additional potential for antioxidants as modulators of CD40L-induced inflammation and platelet activation. In this light, *in vitro* experiments demonstrated inhibition of CD40L-induced clotting system activation by vitamin C [64].

It would be worth testing additional antioxidants, alone or in combination, to further elucidate the efficacy of this novel therapeutic approach in terms of reduction of both CD40L expression and CD40L-induced detrimental cascade.

Antiplatelet agents

As platelets have been shown to be the main contributors to sCD40 shedding, the most obvious approach to down-regulate this mediator seems to be the use of antiplatelet agents such as aspirin. Neither Aukrust et al. [21] nor Cipollone et al. [25] found any significant association between aspirin use and serum levels of sCD40L. These findings are in agreement with the observation that aspirin administration did not attenuate the increased risk of future cardiovascular events associated with high sCD40L levels in healthy women [94]. Aspirin treatment for 7 days in volunteers resulted in a 50% decrease in the release of sCD40L from collagen-induced platelet aggregates *in vitro*, suggesting that TXA_2 is a necessary costimulator of platelets [8]. Recently, to further characterise the platelet origin of plasma CD40L, the dose and time dependence of the effects of aspirin in T2DM was investigated [54]. A randomised, parallel group dose-response study of three different doses (30, 100, 325 mg/day) of aspirin was performed in 18 T2DM patients. Plasma CD40L was significantly reduced by about 40–50% after 7 days of aspirin treatment, with no apparent dose effect, with a slow pattern of recovery over the 10-day wash-out period. The reduction in plasma sCD40L levels as well as the time course of recovery strongly support the hypothesis that in the setting of T2DM both TXA_2-dependent and -independent mechanisms of platelet activation contribute to enhanced release of sCD40L [54]. Thus, despite aspirin use, more potent platelet inhibitors may be required to inhibit the enhanced release of sCD40L.

Clopidogrel, a potent inhibitor of ADP-induced platelet aggregation, has been reported to block ADP-induced CD40L expression when administered for 7 days to healthy volunteers, leading to the hypothesis that this inhibition may, at least partially, account for the beneficial effects of this drug [95]. Further evidence suggests that clopidogrel lowers levels of CRP and CD40L in patients undergoing PTCA [96, 97]. Patients with stable CAD randomised to 8 weeks of clopidogrel or placebo showed a significant reduction in sCD40L levels in the clopidogrel group [98]. In patients with ACS, a loading dose of clopidogrel (300 mg) attenuated the agonist effects of ADP and thrombin receptor agonist peptide (TRAP) on platelet secretion, aggregation, and formation of platelet-monocyte and platelet-neutrophil conjugates [99]. Plasma levels of soluble CD40L and P-selectin were also significantly reduced and may all contribute to the clinical benefits of the drug in ACS.

A loading dose of clopidogrel followed by 75 mg/day combined with aspirin in patients with UA undergoing PTCA significantly reduced sCD40L levels at 24 h after stenting and during follow-up [100].

GP IIb/IIIa inhibitors

GP IIb/IIIa inhibitors are an important class of drugs used in the management of UA and non-ST elevation MI, as well as in preventing clot formation during PTCA. Because GP IIb/IIIa receptors are a binding site for sCD40L, it has been hypothesised that they might modulate sCD40L release from platelets. Addition of eptifibatide to platelet-rich plasma inhibited sCD40L shedding from activated platelets [8]. Although maximum inhibition of sCD40L release is achieved at clinical dose of these drugs, suboptimal concentration paradoxically potentiated sCD40L release. This finding may help to explain the negative outcome from the orally available GP IIb/IIIa antagonist clinical trials, where suboptimal doses of drugs were typically used.

The aforementioned study by Heeschen and coworkers [22] better defined the clinical relevance of CD40L inhibition by GP IIb/IIIa antagonists, as it was the first to establish a link between sCD40L levels, clinical outcome and the therapeutic tool. A total of 1265 patients with ACS were randomly assigned to receive intravenous abciximab or placebo 18–24 h prior to PTCA. Among patients with the highest sCD40L levels, a significant decrease in the incidence of death or nonfatal MI during the 6-month follow-up was observed in subjects receiving abciximab, as compared to those receiving placebo. In contrast, among patients in the lowest quintiles for sCD40L, no significant benefit was observed with the use of abciximab. Thus, sCD40L levels at baseline may predict both in the short-

and long-term the efficacy of abciximab in improving the rate of cardiovascular events in patients with ACS. The authors did not measure sCD40L levels after abciximab treatment as the trial was not designed to show an effect of the drug on sCD40L plasma levels.

Tirofiban was reported to non-significantly limit the increase in sCD40L after PTCA in patients with stable CAD [101]. Recently, to investigate the effects of abciximab and eptifibatide on sCD40L, 98 ACS patients undergoing PTCA were studied [102]. Eighteen to 24 h after PCI, sCD40L was unchanged in the controls, and reduced 30% in the abciximab-treated group and 9% in the eptifibatide-treated group.

Influence of pre-analytical and analytical factors on sCD40L measurement

Although sCD40L is emerging as a promising marker of thrombotic risk, relatively little attention has been paid to the potential impact of pre-analytical and analytical interferences that may confound interpretation of sCD40L measurements [103–108]. Sampling methods and temperature, in fact, profoundly affect the sCD40L assay [105, 109]. For example, post-harvesting activation of platelets when obtaining serum might account for the higher sCD40L levels found in serum compared to plasma [105]. This finding, together with the close correlation of serum sCD40L with platelet count, supports the possibility that serum sCD40L actually reflects platelet CD40L content [105]. Timing of sample processing in relation to blood collection is another important determinant of sCD40L blood levels [106].

In this light, earlier studies in which serum samples were employed should be interpreted with caution, and the use of serum should be encouraged if the objective is to measure the total pool of sCD40L, including both freely circulating sCD40L and CD40L expressed in platelets, monocytes and T cells [105], but not *in vivo* levels of this cytokine [106]. If this is the case, platelet-depleted citrated plasma samples, processed promptly after collection to minimise *ex vivo* release of sCD40L, should be the preferred sample type [108–110]. Finally, it should be emphasised that sCD40L is actually a pool of free soluble and microparticle-bound (mp-CD40L) forms, the proportion of which is highly variable in each individual [105, 111]. Presently, most protocols used in clinical studies are unable to distinguish either form. Therefore, it is of utmost importance to refine and standardise sCD40L measurement beginning with sample handling techniques such as centrifugation force and time, anticoagulant choice and filtration methods. Once this goal is achieved, we will be able to accurately define the role of sCD40L as a prognostic marker of thrombotic risk.

Concluding remarks

CD40L appears as a multiplayer of several cell types in the inflammatory network.

The peculiarity of CD40L as an inflammatory mediator derived from platelets expands the functional repertoire of platelets from players of haemostasis and thrombosis to powerful amplifiers of inflammation by promoting the release of cytokines and chemokines, cell activation and cell–cell interactions.

Thus, compelling evidence indicating the expression of the CD40/CD40L dyad on many different cell types substantiates the role of this pathway in an extraordinary plethora of biological effects ranging from inflammation, endothelial dysfunction and platelet/coagulative activation. The multifunctional role of CD40L as a simultaneous activator of all these systems further blurs the intricate relationship between such events both in the physiological systems and the pathological derangement occurring in atherothrombosis.

Studies performed so far do not have the power to establish the causal role of CD40L in the pathogenesis of the above-mentioned disease states, but these findings raise the question whether elevated sCD40L levels are a reflection of the pathogenetic role of CD40L in the inflammatory and thrombotic processes or whether they are a mere consequence of the clinical event, namely the result of platelet release after thrombus formation. Even in the latter case, CD40L elevation may constitute a reliable marker of platelet activation and of unstable, prone to rupture plaque, and hopefully, as a predictor of cardiovascular event risk. In this light the availability of an inexpensive, commercially available assay to measure sCD40L levels in several clinical settings may help to identify subjects at major risk of events who may benefit most from more aggressive preventive interventions.

The relationship between sCD40L and global risk assessment remains unclear. In a prospective study increased sCD40L seems to be predictive for future cardiovascular events in healthy women [94]. However, in a large cohort of healthy volunteers, sCD40L levels poorly correlate with both the individual components and the calculated Framingham Coronary Heart Disease Risk Score [112], suggesting the need for long-term follow-up studies to answer the question whether the predictive value of sCD40L is independent of the Framingham based global risk assessment.

Another issue is the association between sCD40L levels, atherosclerotic risk factors and subclinical atherosclerosis. In large and representative multiethnic population studies such as the Dallas Heart studies, sCD40L was not associated with most atherosclerotic risk factors and with subclinical atherosclerosis [113], suggesting that sCD40L is not a reliable tool for screening of early atherosclerosis

in the general population. It is possible to hypothesise that the validity of sCD40L elevation is proportional to the extent of platelet activation in each setting, but this assumption requires further studies.

The key research priorities for the near future will focus on determining the relevance of sCD40L as a putative therapeutic target. Several studies have already shown the ability to lower CD40L levels by several agents, suggesting its potential role as a reliable tool to monitor the efficacy of antiplatelet, antidiabetic or lipid-lowering drugs. Whether lowering the levels of this molecule translates into an improvement of cardiovascular outcomes remains to be elucidated.

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