

Review Article

Membrane-associated CD40L and sCD40L in atherothrombotic disease

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Summary

Recent data suggests that CD40L is involved in the pathogenesis of atherothrombotic disease. This review will focus on the history of CD40L, its role in platelet-mediated pathogenesis of

atherothrombotic disease, its association to clinical syndromes and procedures, and its role in determining plaque rupture.

Keywords

CD40L, atherothrombotic disease, platelets, extracellular matrix, matrix metalloproteinase

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Introduction

In recent years, the concepts underlying the pathogenic mechanisms of atherothrombosis have evolved to incorporate the interaction between CD40 and CD40L. Studies suggest this dyad may constitute an important mediator of vascular inflammation and therefore may serve as a molecular link between inflammatory processes, atherosclerosis, and thrombosis. The following review will focus on the role CD40L and sCD40L play in the processes preceding the onset of acute coronary syndromes (ACS): atherogenesis, thrombosis, vascular inflammation and extracellular matrix (ECM) degradation.

History of CD40 and CD40L

CD40 is an integral membrane protein belonging to the tumor necrosis factor (TNF) receptor superfamily (Fig. 1). Its multi-potent immunomodulating ligand, CD40L (termed gp39 (1) and

recently renamed CD154), is a trimeric, type II transmembrane member of the same superfamily (Fig. 1).

Originally thought to be restricted to B lymphocytes, dendritic cells, and basal epithelial cells (2), CD40 is now known to be present on a wide array of cells, both atheroma and non-atheroma associated (3-5). Its ligand, CD40L, was first identified in activated CD4+ T cells (6-8), mast cells (9), polymorphonuclear granulocytes (9), and natural killer cells (10). Subsequent studies revealed functional CD40L expression in a myriad of cell types, namely human endothelial cells (ECs) (11), smooth muscle cells (SMCs) (11), macrophages (11), and platelets (12). CD40/CD40L ligation-mediated signaling was initially thought to occur when CD40 trimerized to bind one CD40L molecule inducing CD40-associated factor (CRAF) to associate with the CD40 cytoplasmic domain (Fig. 2) (13). However, recent evidence suggests that CD40 does not oligomerize into a trimeric receptor by binding a ligand trimer, but rather is membrane-surface bound as a preformed trimer com-

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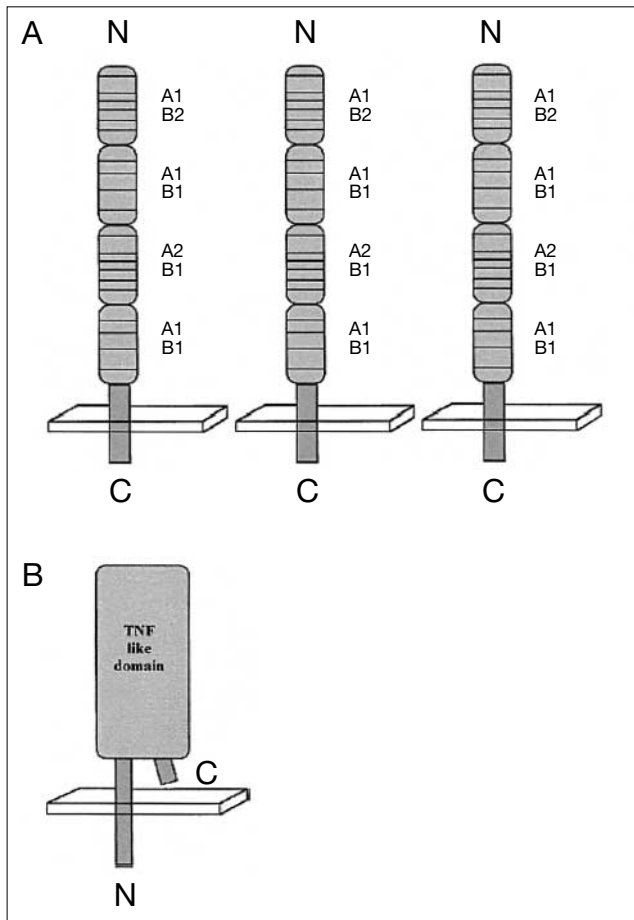


Figure 1: Structure of the CD40 receptor: (A) Schematic representation of the CD40 protein, which is a type I transmembrane receptor. The extracellular region of CD40 is cysteine-rich (20 residues), as indicated by the horizontal lines. Initially, four cysteine-rich domains were identified. Subsequent studies have suggested that every domain can be divided into two cysteine modules (A1, A2, B1, B2), as indicated. (B) Structure of the CD40L molecule. Schematic representation of the CD40L protein, which is a type II membrane protein with an intracellular amino terminus and an external carboxy terminus. The extracellular region of CD40L shows structural homology with other members of the TNF family.

plex (Fig. 2) (14). The latter model is supported by data showing conserved, extracellular pre-ligand-binding assembly domains (PLAD) mediate ligand-independent assembly of trimerized receptors (14). Further support for the PLAD model comes from data demonstrating greater biological activity for the trimeric recombinant CD40L as compared to the monomeric form (15). Originally, CD40/CD40L-dependent signaling was thought to play a role only in humoral (16) and cell-mediated immune responses (17). However, recent studies have demonstrated that CD40/CD40L interactions in atheroma-associated cells, platelets, and endothelium are involved in a broad range of biological functions, including atherogenesis, thrombosis, inflammation, and ECM degradation.

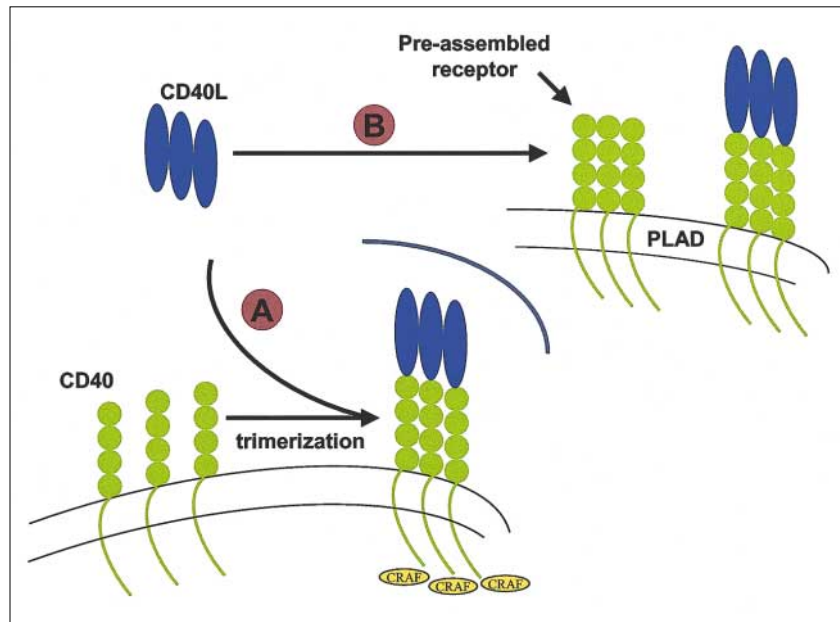
Laying the foundation for CD40L in atherogenesis and thrombosis

Early evidence pointed to the involvement of inflammatory and immune-mediated mechanisms in the pathogenesis of atheroma (18). T lymphocytes in human plaque were known to stain positively for CD40L. The role of CD40L in lesion progression and thrombosis was established through gene-targeting studies utilizing murine knockout models. In low-density lipoprotein knockout mice, treatment with an anti-CD40L monoclonal antibody (mAb) significantly reduced atherosclerotic lesion size and macrophage, T lymphocyte, and lipid content (19). Schonbeck et al utilizing the same murine model in a temporally-longer study not only demonstrated that anti-CD40L mAb limited atherosclerotic disease evolution, but also conferred lesion characteristics suggesting a stable plaque (20). In another exciting experiment, both ApoE and CD40L gene deletions demonstrated a significant reduction in plaque area and cellular content in advanced lesions leading to a stable plaque phenotype (21). Moreover, CD40L deficiency was shown to protect against microvascular thrombus formation, while recombinant soluble CD40L (sCD40L) restored normal thrombosis (22). These initial studies provided evidence that suggested a significant role for CD40L in atherothrombotic disease.

Platelet-associated CD40L and sCD40L

Recently, much attention has been focused on CD40L's cryptic existence in platelets and its potential role in mediating a platelet-dependent inflammatory response associated with the atherothrombotic state. Pioneering work has shown platelet-associated CD40L to elicit an inflammatory response from ECs (12) and induce human monocyte (23), endothelial (24, 25) and vascular smooth muscle (26) tissue factor (TF) expression in a CD40/CD40L-dependent manner. Furthermore, Henn et al have demonstrated that upon platelet stimulation, CD40L is expressed on the surface and then subsequently cleaved to generate a soluble, trimeric fragment, sCD40L (Fig. 3) (4). Though no definitive data identifies platelets as the sole source of sCD40L found in circulation (4, 27) platelet counts (28) and platelet activation (29) have been shown to correlate with sCD40L. Although ambiguity exists regarding the exact mechanism for sCD40L generation, it has been postulated that sCD40L of T lymphocyte or platelet provenance is generated by proteolytic cleavage mediated through the CD40/CD40L-dependent activation of a presently unidentified membrane-bound protease (4, 28, 30). Interestingly, sCD40L maybe involved in a self-perpetuating feedback loop, whereby this soluble ligand binds platelet-bound CD40 and leads to further proteolysis of platelet-associated CD40L, and thus further generates sCD40L (4). Additionally, sCD40L is able to ligate plate-

Figure 2: Models of CD40/CD40L-mediated signaling. (A) Schematic representation of CD40/CD40L induced CRAF-mediated signaling. This model incorporates the concept of ligand induced CD40 receptor trimerization. (B) Schematic representation of the PLAD model of CD40-CD40L signaling. This model involves the notion that the CD40 receptor is not assembled through ligand induced trimerization, but in fact is present as a preformed trimer complex. CRAF indicates CD40-associated factor. PLAD indicates pre-ligand-binding assembly domains.



let glycoprotein (GP) IIb/IIIa complex via its lysine-arginine-glutamic acid (KGD) motif to confer a thrombogenic proclivity, and through this mechanism may play a role in high shear dependent platelet aggregation (22).

There is conflicting data whether sCD40L elicits an inflammatory response. A recombinant form of sCD40L upregulates the endothelial surface expression of adhesive proteins (31). Additionally, recombinant sCD40L has been identified in augmenting chemokine production from peripheral blood mononuclear cells (27, 32) and inducing pulmonary inflammation (33). Recently, however, natural sCD40L was shown to be incapable of inducing an inflammatory response from ECs in vitro (4). These inconsistencies may relate to the fact that the structural differences between recombinant and natural forms of sCD40L confer fundamentally different stability and biologic activities. The difference in protein structure may be predicated at the post-translational level, possibly attributable to an aberration caused by the cell system utilized to express sCD40L, i.e. bacterial vs. mammalian cells. Yet another plausible explanation for the observed difference in provoking an inflammatory response may simply be contamination of the purified protein.

Available evidence regarding the role of sCD40L in vascular endothelial growth factor (VEGF)-mediated EC regeneration presents another conundrum. An interesting study by Melter et al has demonstrated that sCD40L not only induces the expression of VEGF in ECs and monocytes, but that VEGF regulation is under CD40-dependent transcriptional control (34). In addition, this study also demonstrates that EC treatment with sCD40L leads to proliferation through a VEGF-dependent mechanism (34). This latter finding defines a protection process whereby eroded plaque or denuded endothelium can be reendothelialized. In contrast, it has recently been shown that

recombinant CD40L as well as membrane-associated CD40L abrogates VEGF-induced EC migration (35). Although a precise explanation for these controversial findings remains unclear, one may speculate that part of the answer lies within the context and nature of the experiments performed (modified EC growth assay vs. scratched wound assay) and structural conformation of the CD40L molecule utilized (recombinant or membrane-associated CD40L vs. sCD40L).

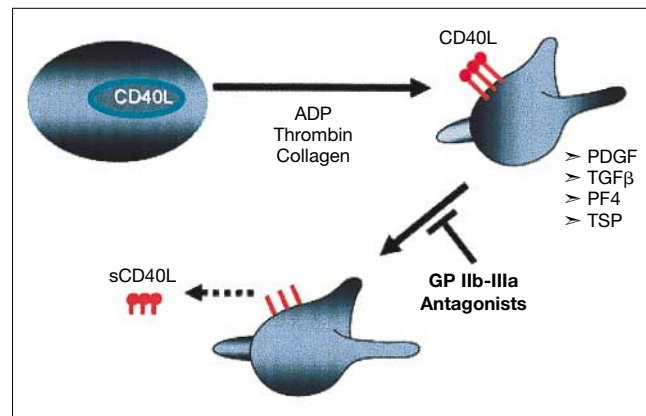


Figure 3: The shedding of soluble CD40L during platelet stimulation. CD40L is cryptic in unstimulated platelets but rapidly translocates to the platelet surface when platelets are activated by agonists such as adenosine diphosphate (ADP), thrombin, or collagen. The translocation of CD40L seems to coincide with the release of α -granule contents including platelet-derived growth factor (PDGF), transforming growth factor beta (TGF β), platelet-factor 4 (PF4), and thrombospondin (TSP). The surface-expressed CD40L is cleaved and shed from the platelet surface in a time-dependent manner as sCD40L. GP IIb/IIIa antagonists block the hydrolysis and subsequent release of sCD40L from platelets.

Clinical Syndrome/ Procedure	Platelet CD40L	T Lymphocyte CD40L	Monocyte CD40	sCD40L
ACS	↑	**	**	↑; (+) correlation to sICAM-1 and sVCAM-1
UA	↑	↑	**	↑
MI	↑	**	**	↑
Hypercholesterolemia	↑	**	↑	↑, (+) correlation to FVIIa and prothrombin f1.2
Diabetes with CAD	**	**	**	↑
CPB	**	**	**	↑
PTCA	**	**	**	↑; high risk for death/non-fatal MI in pts post-PTCA
VBT	↑	**	**	**
Predictor/Marker	**	**	**	↑; high risk of cardiovascular events in ♀

Listed are the raised levels of the corresponding cell-surface expressed CD40/CD40L or sCD40L in its respective syndrome or procedure. Arrow (↑) indicates enhanced level or expression of the CD40 or (s)CD40L molecule. Double star (**) indicates there is no data currently available with regard to the respective CD40 or (s)CD40L syndrome/procedure association.

ACS = acute coronary syndrome; UA = unstable angina; MI = myocardial infarction; CAD = coronary artery disease; CPB = cardiopulmonary bypass; PTCA = percutaneous coronary transluminal angioplasty; VBT = vascular brachytherapy; sICAM-1 = soluble intracellular adhesion molecule 1; sVCAM-1 = soluble vascular cell adhesion molecule 1; ♀ = women.

Table 1: Elevated levels of CD40, CD40L, and sCD40L in clinical syndromes and procedures.

CD40L and sCD40L both consist of homologous, multi-functional structural domains. They both bind CD40 by way of their TNF homology domain (36-38) to induce cellular signaling. Moreover, they both bind the platelet glycoprotein IIb/IIIa receptor through their KGD peptide sequence and may stabilize arterial thrombi in this manner (22). GP IIb/IIIa receptor antagonists at clinically relevant doses inhibit platelet release of sCD40L in vitro (39). Furthermore, subtherapeutic dosing of these potent antiplatelet agents is responsible for potentiating the release of the soluble ligand (39). Interestingly, both GP IIb/IIIa-dependent platelet adhesion to endothelium and GP IIb/IIIa engagement upregulate platelet-associated CD40L (40). These data suggest a significant role for the GP IIb/IIIa receptor in modulating the platelet-CD40L system.

CD40, CD40L and sCD40L in the clinical domain

Raised levels of cell-associated CD40L and sCD40L have been associated with a variety of clinical syndromes (Table 1). Elevated levels of sCD40L have been reported in patients with unstable angina (UA) (27, 41), myocardial infarction (MI) (41), hypercholesterolemia (42, 43), and diabetes with angiographically proven coronary artery disease (44). Furthermore, increased levels of platelet-associated CD40L were found in patients suffering from UA and MI (41). Increased expression

of CD40L on T lymphocytes has also been demonstrated in patients with UA (27). In addition, elevated levels of platelet-associated CD40L and monocyte CD40 have been shown in patients with hypercholesterolemia (42). Elevated levels of the hydrolyzed ligand are also correlated with soluble leukocyte adhesion molecules in ACS patients (45) and prothrombotic molecules in hypercholesterolemia (46), suggesting a role for sCD40L in the chronic inflammation and thrombogenicity associated with these conditions.

Procedures such as cardiopulmonary bypass (47) and percutaneous transluminal coronary angioplasty (PTCA) (27) have been shown to induce sCD40L release. Furthermore, patients undergoing vascular brachytherapy adjunct to PTCA demonstrated enhanced levels of platelet-associated CD40L (48). Patients undergoing high resolution magnetic resonance imaging of carotid atheroma with evidence of an intraplaque lipid pool have demonstrated elevated plasma levels of sCD40L (49). A prospective study demonstrated that raised sCD40L portends an increased risk factor for future cardiovascular events in healthy women (50). Additionally, a recent substudy from the c7E3 Fab Antiplatelet Therapy in Unstable Refractory Angina (CAPTURE) Study group showed an increased risk for death and non-fatal MI in patients with elevated sCD40L levels undergoing PTCA (29). This study demonstrated that platelet inhibition by pretreatment with abciximab, a potent platelet glycoprotein IIb/IIIa receptor antagonist, markedly reduced the

incidence of death and non-fatal MI in these patients (29). These studies illustrate a clear association for (s)CD40L in clinical syndromes and procedures and obviate the need for clinical investigations to further elucidate the function of CD40L in the *in vivo* pathogenesis of the atherothrombotic state.

Lipid lowering and oxidized low density lipoprotein effects on CD40, CD40L, and sCD40L

Lipid lowering interventions, whether dietary or pharmacologic, appear to be important mediators in reducing CD40, CD40L and sCD40L levels *in vitro* and *in vivo*. Dietary lipid lowering has been shown to significantly decrease CD40 and CD40L in rabbit atheroma (51). Statins have demonstrated remarkable immunomodulating properties. They have been shown to downregulate CD40 and CD40L expression in human ECs (52, 53). Furthermore, statin therapy in hypercholesterolemic patients decreased sCD40L (43, 46), with concomitant reduction in Factor VIIa and prothrombin $f_{1,2}$, a cleavage product mediated by the Factor Xa-dependent proteolysis of prothrombin to thrombin (46). This data suggests that the soluble ligand may serve as the molecular link between hypercholesterolemia and the prothrombotic state. Additionally, patients with moderate hypercholesterolemia being treated with a HMG-CoA reductase inhibitor demonstrated downregulation of monocyte surface-expressed CD40 (42). Studies have also shown oxidized low-density lipoprotein to increase both CD40 and CD40L expression in human venous ECs (52) and coronary artery ECs (54) in a concentration-dependent manner. These investigations suggest that administration of statins to hypercholesterolemic patients may not only reduce lipid levels, but may also provide protection from untoward CD40/CD40L-mediated cardiovascular sequelae, and impart evidence to necessitate addressing oxidative stress in this patient population.

Atheromatous plaque stability mediated by CD40L-dependent ECM degradation

There is evidence to suggest a major role for CD40/CD40L interactions in determining the propensity for atheromatous plaque to rupture. Increased expression of matrix metalloproteinases (MMP), in particular MMP-1, MMP-3, MMP-9, and activated MMP-2, is induced by immune-mediated CD40L on human vascular SMCs (Fig. 4) (55). Furthermore, T lymphocyte-associated CD40L has been shown to activate MMP-2, induce the *de novo* synthesis and release of MMP-9, and upregulate the expression of MMP-1 and MMP-3 in human vascular ECs (56). Ligation of monocyte/macrophage CD40 by CD40L induces MMP-1 and MMP-3 expression on these cell types (57). Data suggest that CD40L ligation of human vascular

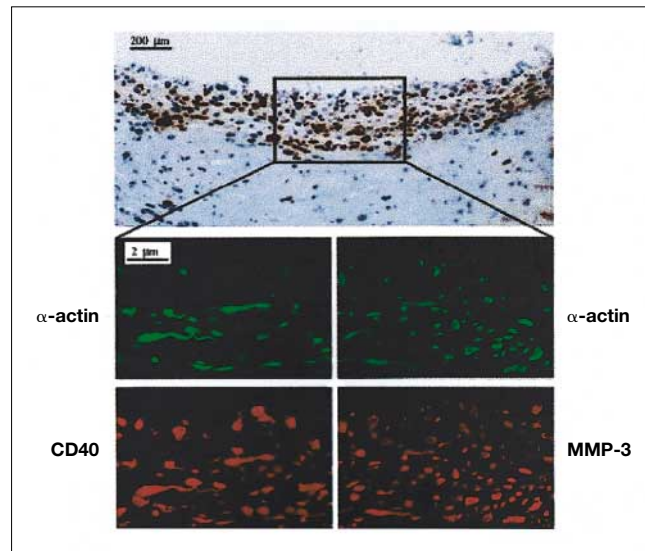


Figure 4: Colocalization of CD40-positive SMCs with stromelysin (MMP-3) in the fibrous cap of a coronary lesion *in situ*. Top, Low-power view ($\times 40$) of a paraffin-fixed section of a human coronary artery plaque shows the accumulation of SMCs in the fibrous cap of the lesion. Middle and bottom, High-power views ($\times 400$) combined with the immunofluorescence double-labeling technique revealed colocalization of CD40 (left panels) and MMP-3 (right panels) on SMCs using an anti- α -actin (FITC-conjugated) antibody (middle) and an anti-CD40 or anti-MMP-3 (Texas red-conjugated) antibody (bottom). The lumen of the artery is at the top of the photomicrograph. Similar results were obtained in four atherectomy specimens.

SMCs results in the loss of interstitial (fibrillar) collagen, the structural and load-bearing molecule of the plaque fibrous cap (58, 59), by CD40L-mediated modulation of MMP activity (60). Loss of this ECM protein is therefore especially noteworthy because it may confer an increased proclivity towards plaque rupture (61). Furthermore, platelet-associated CD40L upregulates the expression of membrane-anchored protease receptors, urokinase-type plasminogen activator receptor (uPAR) and membrane type-1 matrix metalloproteinase (MT1-MMP) on human umbilical vein ECs (40). uPAR and MT1-MMP bind and induce the ECM degrading activity of urokinase-type plasminogen activator (uPA) and MMP-2, respectively (62, 63). In addition, platelet-associated CD40L enhances endothelial secretion of uPA, tissue-type plasminogen activator, and MMP-1 and proteolytic activity in MMP-2 and MMP-9 (40). Of potential clinical relevance, activated platelet-induced MMP-9 of endothelial provenance is reduced by anti-GP Ib and anti-GP IIb/IIIa antibodies, and platelet GP IIb/IIIa antagonists (40). Interestingly, in this study a similar observation is made with anti-CD40L antibody suggesting that effective and continuous platelet inhibition by GP IIb/IIIa antagonists may attenuate MMP expression in a platelet-CD40L dependent manner (40). Recently, CD40L has been implicated in inhibiting nitric oxide (NO)-dependent EC migration by augmenting production of

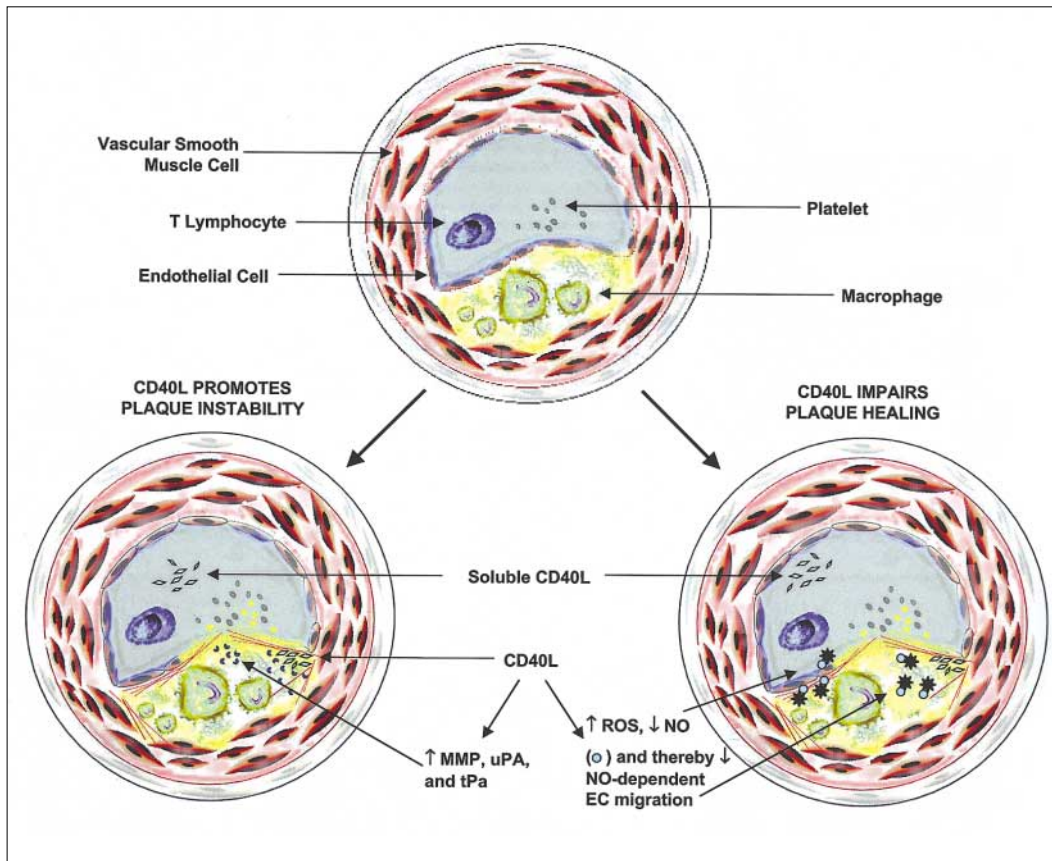


Figure 5: Dual action of CD40L in plaque destabilization. Ligation of CD40 by CD40L upregulates the expression and extracellular matrix degrading activity of a myriad of MMPs in human vascular smooth muscle cells, endothelial cells, and monocyte/macrophages. CD40/CD40L interactions further increase the propensity for plaque rupture by upregulating the endothelial expression of uPA and tPA whose cleavage product, plasmin, is known to activate MMPs. In addition, NO-mediated EC migration to reendothelialize the disrupted vessel wall is prevented by the CD40L-mediated neutralization of NO by ROS. MMP = matrix metalloproteinases; uPA = urokinase-type plasminogen activator; tPA = tissue-type plasminogen activator; NO = nitric oxide; ROS = reactive oxygen species.

reactive oxygen species that neutralize NO (35). This novel concept in addition to the data reported above reveals the dual nature in which CD40L may prevent the stabilization of rupture-prone atheromatous plaque by upregulating ECM degrading enzymes and their activity as well as preventing endothelial regeneration after plaque erosion and/or mechanical arterial injury (Fig. 5). Taken together, these data highlight the crucial role the CD40/CD40L signaling dyad plays in undermining the mechanical integrity of coronary atheromata.

Concluding remarks and unanswered questions

Investigations over the years have yielded data implicating a myriad of pathways and mediators involved in atherothrombotic disease. Much attention, however, has recently been focused on CD40L. Increasing evidence suggests that CD40L, whether membrane-bound or in soluble form, may be a pivotal link between the underlying mechanisms leading to the acute

complications observed in this disease state. As discussed above, the ligand's involvement in atherogenesis, thrombus formation, platelet-mediated inflammation, and plaque destabilization as well as elevated levels in a slue of clinical syndromes make it a desirable target for pharmacologic intervention. Can the presumed *in vivo* pleiotropic effect of CD40L be attenuated by intervening proximally, say before expression or release from its provenance. Standard platelet GP IIb/IIIa inhibitors may be able to confer such a possible therapy. Can other anti-platelet medications do the same, namely clopidogrel or aspirin? Furthermore, can direct thrombin inhibitors, be they univalent or bivalent, provide protection from platelet-associated CD40L or sCD40L mediated thrombotic events? And interestingly, can the platelet-activating effects of heparin augment platelet-CD40L or sCD40L mediated pathologies. Studies to investigate these questions and future discoveries may lead to the development of novel, targeted therapies to modulate the CD40L-mediated clinical sequelae of atherothrombosis predicated on receptor antagonism or attenuating ligand active site functional activity.

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