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Elevated release of sCD40L from platelets of diabetic patients by thrombin, glucose and advanced glycation end products

NEREA VARO, PETER LIBBY, REBECCA NUZZO, JOSEPH ITALIANO, ALESSANDRO DORIA, UWE SCHÖNBECK

Abstract

The pro-inflammatory CD40/CD40L dyad participates in atherogenesis. Plasma levels of the soluble ligand (sCD40L) predict cardiovascular events and are elevated in diabetic patients. This study compared CD40/CD40L surface expression on platelets and T lymphocytes of diabetic and control subjects, and tested whether glucose and advanced glycation end products (AGEs) stimulate sCD40L release.

Constitutive and inducible surface expression of CD40/CD40L on platelets or T lymphocytes did not differ between diabetic patients (n=9) and controls (n=13). Platelets from diabetic patients contained higher intracellular CD40L than controls ($p < 0.05$) and thrombin stimulated greater platelet sCD40L release in diabetic patients (15.11 ± 16.77 ng/ml) compared to controls (3.64 ± 2.03 ng/ml; $p < 0.05$). Glucose and AGEs induced platelet sCD40L release and CD40L expression in mouse megakaryocytes.

This study demonstrates elevated CD40L content and inducible release from platelets of diabetic patients, and identifies glucose and AGEs as potential triggers of expression and release accounting for the elevated sCD40L plasma levels in these patients.

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Key words: CD40 ligand, inflammation, diabetes, platelets.

Introduction

The inflammatory mediator CD40L and its receptor, CD40, participate in the pathogenesis of atherosclerosis. Activation of CD40 on human vascular endothelial and smooth muscle cells, and on mononuclear phagocytes as well, mediates a wide variety of pro-atherogenic functions *in vitro*.^{1,2} Disruption of CD40 signalling in hypercholesterolaemic mice not only inhibits lesion formation but also limits the progression of established atheroma and promotes plaque characteristics associated with stability.^{3–6} Moreover, patients with unstable angina express higher plasma concentrations of the soluble form of CD40L (sCD40L) compared to healthy volunteers or those with stable angina.⁷ Elevated plasma levels of sCD40L precede thrombotic events and identify apparently healthy individuals who are at heightened risk of cardiovascular events.⁸ Furthermore, sCD40L levels predict recurrent cardiovascular complications in patients with acute coronary syndromes^{9,10} and correlate with features of plaque composition such as the presence of a lesional lipid core.¹¹

Elevated levels of sCD40L also associate with known traditional cardiovascular risk factors. Patients with diabetes mellitus have significantly elevated plasma levels of sCD40L compared to non-diabetic controls. Furthermore, treatment with thiazolidinediones (TZDs; glitazones) can diminish plasma sCD40L levels.^{12,13} However, the cellular source of sCD40L and its inducers of release into plasma remain uncertain, and the surface levels of CD40 and CD40L found on peripheral blood cells of diabetic subjects are unknown.

Advanced glycation end products (AGEs) arise by the non-enzymatic glycation of proteins via reducing sugars, such as glucose. Serum of diabetic patients contains elevated levels of AGEs, and AGEs may contribute to the complications of diabetes mellitus, although the underlying molecular mechanisms remain uncertain.^{14,15} AGEs act in part by binding to the receptor for advanced glycation end products (RAGE), which also binds ligands other than AGEs, such as S100/calgranulins.^{16,17} Ligation of RAGE on several cell types implicated in atherosclerosis, such as endothelial cells and mononuclear phagocytes, induces pro-inflammatory responses that might contribute to the evolution of both diabetes and atherosclerosis.¹⁸

The present study compared CD40 and CD40L expres-

sion by platelets and T lymphocytes of diabetic and control subjects and tested the hypothesis that glucose and AGEs stimulate release of sCD40L from these cell types.

Research design and methods

Subjects

The study population consisted of type 2 diabetic patients (five male and four female, aged 58 ± 18 years) followed at the Joslin Diabetes Center (Boston, MA) after diagnosis (according to the recommendations of the American Diabetes Association Expert Committee on the Classification and Diagnosis of Diabetes) and non-diabetic controls (six male and seven female, aged 35 ± 15 years).

Whole blood was collected in 0.1 volume of Aster-Jandl anticoagulant solution (85 mM sodium citrate, 69 mM citric acid) by venous puncture. No significant differences were found between diabetic patients and controls in levels of total cholesterol (216 ± 70 vs. 203 ± 32 mg/dl, respectively [5.59 ± 1.81 vs. 5.25 ± 0.82 mmol/L]), low-density lipoprotein cholesterol (LDL) (137 ± 50 vs. 140 ± 38 mg/dl, respectively [3.54 ± 1.29 vs. 3.62 ± 0.98 mmol/L]) high-density lipoprotein cholesterol (HDL) (49 ± 34 vs. 42 ± 20 mg/dl, respectively [1.26 ± 0.88 vs. 1.08 ± 0.51 mmol/L]), and triglycerides (151 ± 91 vs. 105 ± 44 mg/dl, respectively [1.70 ± 1.07 vs. 1.18 ± 0.49 mmol/L]). Study protocols were approved by the institutional review board and all participants gave their written informed consent.

Reagents

Human thrombin, phorbol-12 myristate 13-acetate (PMA), ionomycin, bovine serum albumin (BSA), D-glucose, mannitol, vitamin C, vitamin E, and butylated hydroxytoluene (BHT) were obtained from Sigma (St. Louis, MO). Anti-human CD40L, anti-CD40 and control IgG1 fluorescein isothiocyanate (FITC)-conjugated antibodies were obtained from Ancell (Bayport, MN). FITC-labelled P-selectin was purchased from Pharmingen (San Diego, CA). The thrombin receptor PAR-1-activating peptide (TRAP), S100 calgranulin, and rCD40L were purchased from Bachem (San Diego, CA), Calbiochem (San Diego, CA), and Leinco (St. Louis, MO), respectively.

Generation of AGE-BSA

BSA was incubated with glucose (0.5M) in phosphate-buffered saline (PBS) under sterile conditions for eight weeks at 37°C . During the incubation period, fluorescence intensity was determined (excitation 370 nm, emission 440 nm) to monitor the formation of AGEs. At the end of the incubation time, the presence of AGEs was confirmed by a change to brown colour, a shift in 2D gel electrophoreses, and Western blot analysis employing an anti-AGE antibody (TransGenic, Inc.[Kumamoto, Japan]) (data not shown). Samples were dialysed against PBS and stored at -20°C , protected from light.

Cell culture

Platelets were isolated from platelet-rich plasma generated by centrifuging whole blood containing Aster-Jandl anticoagulant solution (200 xg, 20 minutes) by a metrizamide gradi-

ent¹⁹ and resuspended in 140 mmol/L NaCl, 3 mmol/L KCl, 0.5 mmol/L MgCl_2 , 5 mmol/L NaHCO_3 , 10 mmol/L glucose, 10 mmol/L N-2-hydroxyethyl piperazine-N 2-ethanesulfonic acid (HEPES), pH 7.4. The concentration of platelets was adjusted to $2 \times 10^9/\text{mL}$ in the buffer and purified platelets were allowed to rest (30 minutes, 37°C). For the experiments employing glucose, platelets were isolated glucose-free by layering platelet-rich plasma onto a Sepharose 2B column, eluted with HEPES buffer, 0.3% BSA, and adjusted to $2 \times 10^9/\text{mL}$. Platelets were stimulated (30 minutes, 37°C) with human rCD40L (10 $\mu\text{g}/\text{mL}$), thrombin (1U/mL), TRAP (25 μM), D-glucose (range 100 mg/dl [5.5 mM] to 500 mg/dl [27.7 mM]), or S100 calgranulin (100 nM) in the absence or presence of different antioxidants (vitamin E [0.5 mM], vitamin C [100 μM], and BHT [10 μM]) or the glycoprotein receptor IIb-IIIa antagonist eptifibatide (2 $\mu\text{g}/\text{mL}$). Equimolar concentrations of D-mannitol were used as iso-osmotic controls. Ten μL of the platelet solution were collected for fluorescence-activated cell sorting (FACS) analysis. Supernatants were obtained (15 min, 1,000 g) and pellets were resuspended in lysis buffer (1% Triton X-100, 20 mM Tris/HCl [pH 7.4], 150 mM NaCl, 1mM EDTA [pH 8.0]).

Megakaryocytes were isolated from murine fetal livers and single cell suspensions were generated, as described previously.²⁰ Briefly, until the fifth day of culture, cells were incubated with S100 calgranulin (0.1 μM) or glucose (16.6 mM) and placed on a 1.5–3.0% albumin step gradient. Following sedimentation, the enriched population of megakaryocytes was lysed in 1% Triton buffer and separated (50 μg total protein/lane) by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) under reducing conditions before being blotted onto polyvinylidene difluoride membranes (Millipore [Bedford, MA]). Blots were blocked for one hour in 5% non-fat milk/PBS/0.1% Tween 20 and primary antibody was added overnight (1:250 goat anti-mouse CD40L, Santa Cruz [Santa Cruz, CA]). Immunoreactive proteins were visualised after incubation with a secondary antibody (rabbit anti-goat, 1:5000) employing the Western Lighting Chemiluminescence system (Perkin Elmer [Boston, MA]).

T lymphocytes were isolated from whole human peripheral blood by CD4^+ selection employing magnetic beads coated with anti-CD4 mAb (Dynabeads M450 CD4; Dynal [Brown Deer, WI]). The purity of the T-lymphocyte preparations was $\geq 98\%$, as determined by FACS using an anti-human CD4^+ mAb, FITC-conjugated (Pharmingen, San Diego, CA). The cells were cultured for six or 24 hours in RPMI 1640 (BioWhittaker, Walkersville, MD) in the absence or presence of phorbol myristate acetate (PMA) and ionomycin (50 ng/1 $\mu\text{g}/\text{mL}$). CD40 and CD40L cell-surface expression were measured by FACS following six hours of stimulation and sCD40L release was measured by enzyme-linked immunosorbent assay (ELISA) in the supernatants collected after one (no release detected; data not shown) and 24 hours of stimulation.

FACS analysis

Cells were incubated with CD40, CD40L or P-selectin FITC-conjugated antibodies (30 minutes, 4°C). Following

immunofluorescence labelling, cells were washed twice with PBS/0.2% BSA and analysed in a Becton Dickinson FACScan flow cytometer. Data were processed using CellQuest software (Becton Dickinson, Franklin Lakes, NJ). At least 20,000 viable cells per condition were analysed.

ELISA

sCD40L concentrations in plasma as well as culture supernatants and lysates were determined by ELISA (BenderMedSystems [Vienna, Austria] detecting both full length and sCD40L), as described previously.^{13,18} The intra-assay variation among the duplicates for all samples was less than 10%. The detection limit was 10 pg/ml sCD40L.

Platelet-derived growth factor (PDGF) and the chemokine regulated on activation normally T-cells expressed and secreted (RANTES) concentrations were also determined by ELISA (R&D Systems, Minneapolis, MN). The intra-assay variation among the duplicates for all samples was less than 15% for both assays. The detection limits were 15 pg/ml and 30 pg/ml, respectively. Total protein concentration in the lysates was determined by the bicinchoninic acid method (Pierce, Rockford, IL).

Data analysis

Statistical analysis utilised the Statistical Package for Social Sciences (SPSS, 11.0). Results are presented as mean±SD. Means between diabetics and controls were compared using the Mann Whitney test. The Wilcoxon test was used to compare variables before and after stimulation. All p values are two-tailed and all confidence intervals were computed at the 95% level. Multivariable linear regression models were used with sCD40L release after thrombin stimulation as the dependent variable. Age and other possible confounding variables were included as independent factors.

Results

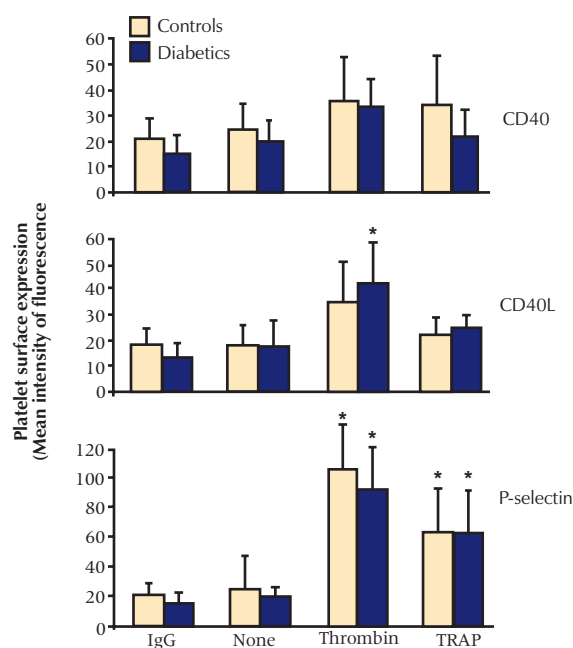
Elevated expression and release of sCD40L by platelets of type 2 diabetic subjects

In accord with our previous report, plasma of type 2 diabetic (n=9) patients contained higher sCD40L levels compared to those in non-diseased (n=13) subjects (2.70 ± 1.1 ng/ml vs. 0.94 ± 0.7 , $p < 0.05$). Neither age nor gender accounted for this difference: this is consistent with observations from previous multivariate analysis in larger cohorts, demonstrating association of elevated plasma sCD40L levels and diabetes mellitus independent of other variables studied, including age and gender.^{12,13}

To test whether these patients also have elevated cellular levels of CD40 and CD40L, we determined surface expression of both the receptor and the ligand on T lymphocytes and platelets and release of sCD40L from these cell types. Surface expression of CD40L on unstimulated T lymphocytes did not differ significantly between type 2 diabetic patients (mean intensity of fluorescence [MFI] 19.0 ± 13.5 ; n=9) and non-diabetic controls (MFI 17.8 ± 9.83 ; n=13), as determined by FACS analysis. Furthermore, inducible cell surface expression of CD40L triggered by stimulation of T lymphocytes with PMA/ionomycin did not differ between

Figure 1. CD40, CD40L and P-selectin surface expression on resting or stimulated platelets does not differ between diabetic patients and controls

Platelets from type 2 diabetic patients (n=9) and controls (n=13) were isolated from whole blood and cultured (30 minutes) in the absence (none) or presence of thrombin (1 U/ml) or TRAP (25 μ M). CD40, CD40L and P-selectin surface expression were determined by FACS. Data are expressed as MFI±SD



* $p < 0.05$ vs. non-stimulated platelets

Key: IgG = immunoglobulin G; TRAP = thrombin receptor PAR-1-activating peptide

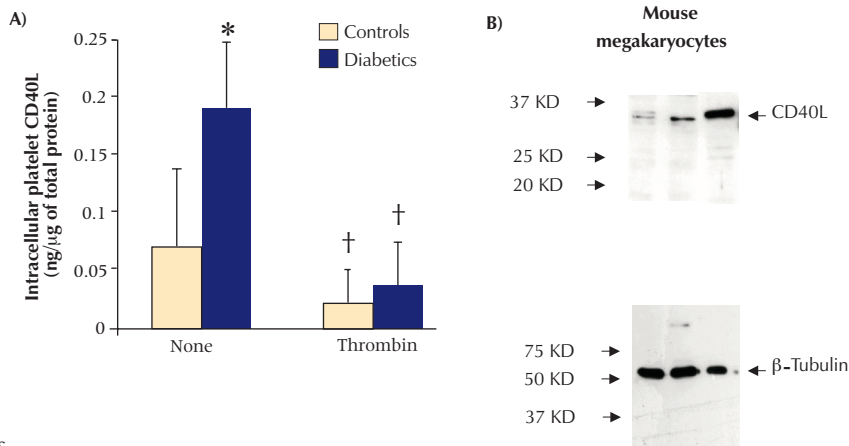
diabetic patients (MFI 35.1 ± 22.8) and controls (MFI 33.9 ± 18.7).

Similarly, cell surface expression of CD40 and CD40L on resting platelets did not differ between type 2 diabetic patients (MFI 20.0 ± 8.2 and 19.8 ± 10.0) and controls (MFI 24.1 ± 10.2 and 20.3 ± 8.5) (figure 1). Stimulation with thrombin (1 U/ml) or TRAP (25 μ M) significantly ($p < 0.05$) increased CD40, CD40L and P-selectin cell surface expression, but comparably so in platelets obtained from diabetic patients and controls. However, platelets from diabetic subjects had significantly ($p < 0.05$) higher resting intracellular concentrations of CD40L compared to controls (0.19 ± 0.05 vs. 0.08 ± 0.05 ng/ μ g of total protein, respectively) (figure 2A); the majority of the ligand localised in the cytoplasm and not within granules (data not shown), as determined by immunofluorescent labelling.

To investigate mechanisms that might explain higher intracellular concentrations of CD40L in platelets from diabetic patients, mouse megakaryocytes were cultured in the absence or presence of S100 calgranulin, an established RAGE agonist (0.1 μ M), or glucose (16.6 mM) (figure 2B). Glucose (figure 2B) and S100 calgranulin (data not shown)

Figure 2. Increased intracellular CD40L content in platelets from diabetic subjects and increased CD40L expression in megakaryocytes by glucose

A) Platelets were isolated from whole blood from type 2 diabetic patients (n=9) and controls (n=13) and incubated (30 minutes) in the absence (none) or presence of thrombin (1 U/ml). Cells were pelleted, resuspended in lysis buffer and CD40L concentrations were determined by ELISA. Data are expressed as ng of CD40L/μg total protein±SD. B) Protein extracts (50 μg) from mouse megakaryocytes stimulated (five days) with S100 calgranulin (0.1 μM) or glucose (16.6 mM) were analysed by Western blotting. Megakaryocytes from three different mice yielded comparable data

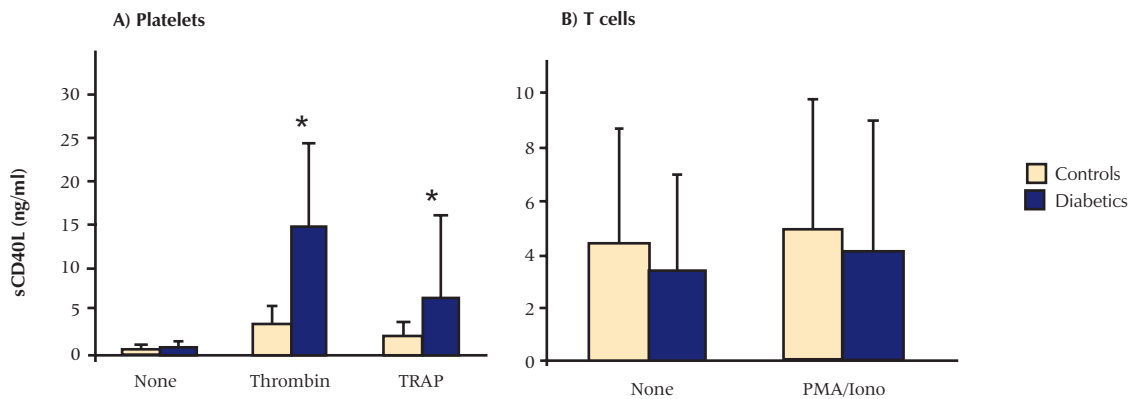


* p<0.05 vs. controls

† p<0.05 vs. non-stimulated platelets

Figure 3. Increased release of sCD40L from platelets, but not T lymphocytes, of diabetic patients compared to controls

T lymphocytes and platelets were isolated from whole blood. T lymphocytes were cultured (24h) in the absence (none) or presence of PMA/ionomycin (50 ng/ml/1 μg/ml); platelets were incubated (30 minutes) in the absence (none) or presence of thrombin (1 U/ml) or TRAP (25 μM) and sCD40L was measured in the supernatants by ELISA. Data are expressed as mean sCD40L±SD



* p<0.05 vs. controls

Key: TRAP = thrombin receptor PAR-1-activating peptide; Iono = ionomycin

induced CD40L expression in mouse megakaryocytes above non-stimulated cells (both n=3). Parallel studies analysing the expression of beta-tubulin immunoreactive protein demonstrated application of equal amounts of protein in each condition.

In accord with the elevated intracellular concentrations, platelets derived from diabetic patients released substantially (p<0.05) more sCD40L than did those from healthy controls following stimulation with the established inducers of sCD40L release, thrombin (1 U/ml; 15.11±16.77 vs. 3.64±2.03 ng/ml) or TRAP (25 μM; 7.26±9.49 ng/ml vs. 2.39±1.87

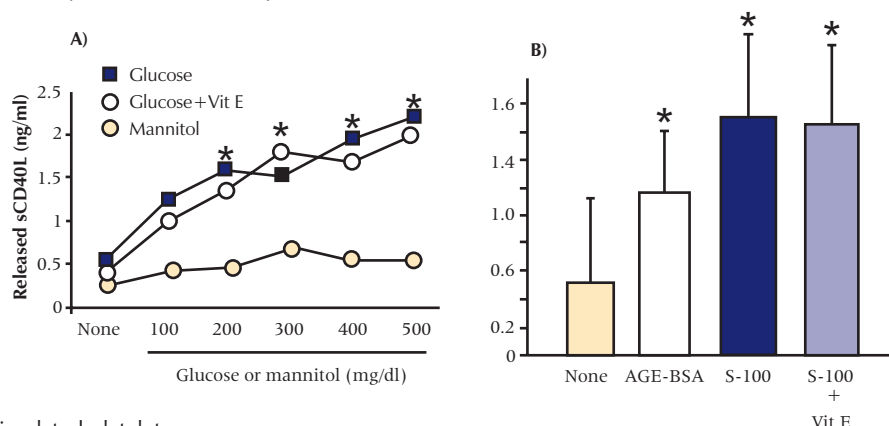
ng/ml) (figure 3A). A significant (p<0.05) decrease in the intracellular content of CD40L in both diabetic patients and controls accompanied the release (figure 2A). Multivariate analysis shows that differences in sCD40L release did not depend on age or other confounding factors. In contrast, release of sCD40L by T lymphocytes obtained from diabetic subjects or controls did not differ significantly (figure 3B).

Glucose, AGEs and S100 calgranulins induce sCD40L release from platelets

Interestingly, stimulation of platelets with glucose signifi-

Figure 4. Glucose, AGE-BSA and S100, but not mannitol, induce sCD40L release from platelets

Platelets isolated from peripheral blood of healthy volunteers (n=6) were stimulated (30 minutes) with (A) increasing concentrations of glucose or mannitol (100 mg/dl [5.5 mM] to 500 mg/dl [27.7 mM]), (B) AGE-BSA (100 µg/ml) or S100 calgranulin (0.1 µM) in the absence or presence of vitamin E (0.5 mM) solubilised in ethanol. Following centrifugation (15 min, 1000 g), sCD40L concentration was determined in the supernatants by ELISA. Data are expressed as mean sCD40L±SD

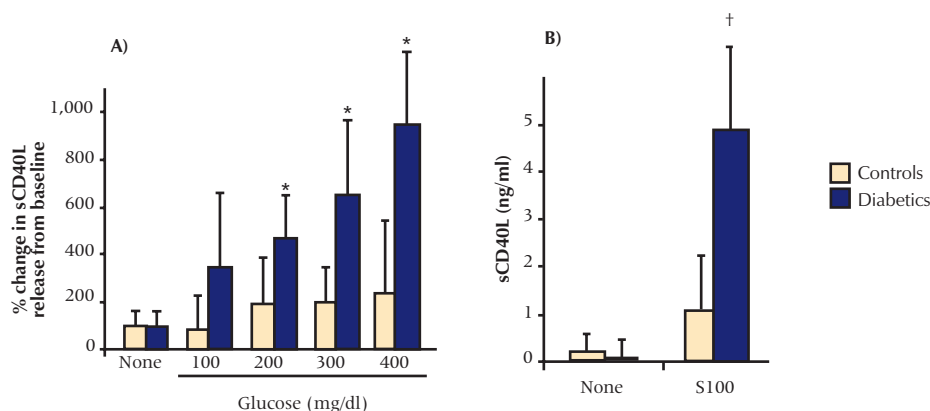


* $p < 0.05$ vs. non-stimulated platelets

Key: Vit = vitamin; AGE-BSA = advanced glycation end products-bovine serum albumin

Figure 5. Glucose- and S100 calgranulin-induced release of sCD40L is higher in diabetic patients compared to controls

Platelets from type 2 diabetic patients (n=9) or controls (n=13) were stimulated with increasing concentrations of (A) glucose (100 mg/dl [5.5 mM] to 500 mg/dl [27.7 mM]) or (B) S100 calgranulin (0.1 µM). Following centrifugation (15 min, 1,000 g), sCD40L concentrations were determined in the supernatants by ELISA. Data are expressed as percent change of sCD40L released in response to glucose compared to baseline in diabetic patients and controls (set as 100%). (B) Data are expressed as mean±SD sCD40L



* $p < 0.05$ vs. controls; † $p < 0.01$ vs. controls

cantly induced P-selectin surface expression (data not shown) and significantly ($p < 0.05$) and concentration-dependently (200 mg/dl [11.1 mM] to 500 mg/dl [27.7 mM]) induced the release of sCD40L (figure 4A). The presence of the antioxidants vitamin E (figure 4A, 4B), vitamin C or BHT (data not shown) did not affect sCD40L release induced by glucose. Equimolar concentrations of mannitol did not affect the release of sCD40L. Furthermore, stimulation of platelets with AGE-BSA (100 µg/ml) or S100 (100 nM) significantly ($p < 0.05$) induced the release of sCD40L (figure 4B).

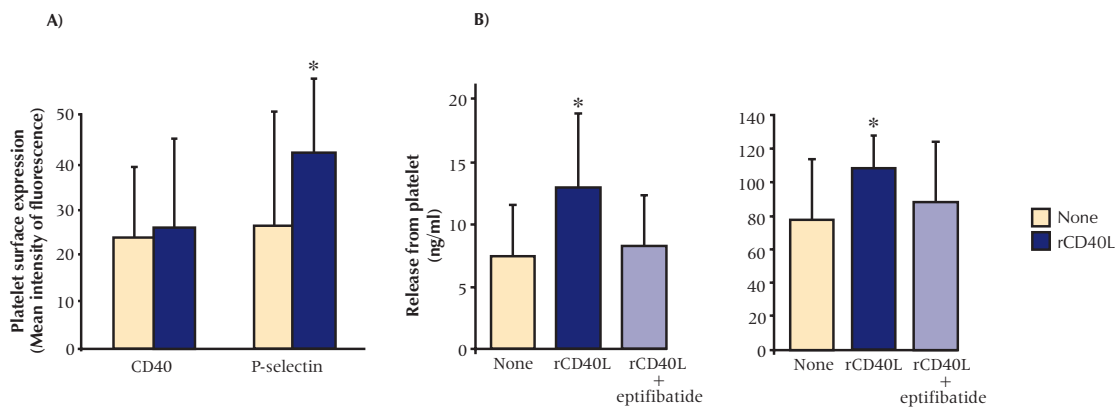
Platelets derived from diabetic patients released significantly more sCD40L than those from controls when stimulated with glucose or S100 calgranulin (figure 5).

Recombinant soluble CD40L increases P-selectin cell surface expression and the release of PDGF and RANTES from platelets

Stimulation of platelets with recombinant CD40L did not affect CD40 cell surface expression (figure 6A). However, stimulation with CD40L significantly ($p < 0.05$) increased platelet P-selectin cell surface expression (mean intensity of fluorescence: 93.4 ± 89.8 and 41.7 ± 31.7 , respectively), compared to non-stimulated platelets (mean intensity of fluorescence: 33.3 ± 54.7 and 26.3 ± 27.8 , respectively). This treatment also significantly ($p < 0.05$) induced the release of other inflammatory mediators, such as platelet PDGF – and RANTES – (13.0 ± 5.8 and 106.2 ± 22.15 ng/ml) compared to non-stimulated platelets (7.5 ± 4.1 and 86.2 ± 34.4 ng/ml,

Figure 6. Recombinant CD40L increases P-selectin surface expression and PDGF and RANTES release by platelets

Platelets were incubated (30 minutes) in the absence (none) or presence of rCD40L (10 μ g/ml). A) CD40 and P-selectin surface expression were determined by FACS and B) PDGF and RANTES concentrations were measured in the supernatants by ELISA. Data are expressed as mean \pm SD



* $p < 0.05$ vs. non-stimulated platelets

Key: PDGF = platelet-derived growth factor; RANTES = (chemokine) regulated on activation normally T-cells expressed and secreted

respectively). The GPIIb-IIIa antagonist eptifibatide blocked PDGF- and RANTES-induced release by rCD40L (8.5 ± 4.3 and 90.2 ± 15.0 ng/ml, respectively), suggesting that rCD40L mediates this proinflammatory response in platelets by binding to GPIIb-IIIa rather than through CD40 ligation (figure 6B).

Discussion

This study explored the mechanisms that underlie elevated plasma sCD40L concentrations in diabetic patients. Several cell types, including T lymphocytes and platelets, may release sCD40L into blood.¹ Platelets release sCD40L upon activation by thrombin *in vitro* and during thrombus formation *in vivo*.²¹ The present study found no difference in the cell surface expression of CD40L on resting T cells or platelets between diabetic patients and controls. Furthermore, sCD40L release from T lymphocytes upon stimulation with PMA and ionomycin was similar in diabetic subjects and controls. Interestingly, however, platelets from diabetic patients released significantly more sCD40L after stimulation with thrombin or TRAP.

Taken together, our data suggest that heightened platelet, rather than T cell, activation likely accounts for the elevated plasma sCD40L concentrations in diabetic patients. Furthermore, induction of CD40L expression in murine megakaryocytes by AGE-modified proteins or hyperglycaemia, both of them relevant to the pathogenesis of diabetes,¹⁵ suggests a mechanism for the observed elevated levels in platelets. These observations shed new light on the thrombotic diathesis of diabetic patients.

Numerous studies have indicated that AGEs contribute to diabetic complications, including nephropathy, retinopathy, neuropathy and vasculopathy.^{17,22} The present study further examined regulation of platelet release of sCD40L by glucose and ligands for RAGE, namely AGE-BSA and S100 calgranulin, as a potential molecular pathway. The observation that antioxidants did not affect glucose- and AGE-induced sCD40L release argues against oxidative stress as

the stimulus of CD40L shedding. Furthermore, the finding that glucose and AGEs, but not mannitol, increased sCD40L release from platelets suggests a novel link between hyperglycaemia and inflammation, and provides a new mechanism for the elevation of sCD40L plasma levels in diabetic patients and the link between diabetes and atherosclerosis. These effects of AGE-BSA and S100 calgranulin probably depend on RAGE, though this proposition was not tested directly in this study. Future studies will need to evaluate whether pharmacological inhibition of AGE formation and disruption of pre-formed AGE protein cross-links can modulate plasma sCD40L levels in diabetic patients.

CD40L translocates rapidly to the platelet surface after platelet stimulation by agonists such as ADP, thrombin or collagen, coincident with the release of alpha granules' content including PDGF, transforming growth factor-beta (TGF- β), PF4 and thrombospondin.²³ Platelets shed CD40L from their surfaces as sCD40L. The present study shows a significant elevation of thrombin- or TRAP-induced CD40L surface expression that did not differ significantly between diabetic patients and controls. However, resting platelets from diabetic patients have increased intracellular content and showed higher release of sCD40L following stimulation compared to controls. These findings suggest that elevated levels of preformed CD40L, but not the pathway(s) of release in platelets rather than T cells, account for the differences in sCD40L plasma concentrations between diabetic patients and control subjects.

Although the functional activity of released sCD40L remains controversial, activated platelets can express and release CD40L, which does elicit pro-inflammatory responses by endothelial cells.²¹ However, little is known regarding the possible effects of CD40L on platelets. Upon activation with different agonists, platelets release from their alpha granules a gamut of preformed pro-inflammatory molecules such as TGF, RANTES, PDGF and P-selectin. We report here the release of two of these mediators following stimulation

with CD40 ligand, RANTES, which stimulates leukocyte chemotaxis and activation,²⁴ and PDGF, a trigger of smooth muscle cell migration and proliferation.²⁵ These findings identify a pathway whereby the activation of platelets by CD40L may link thrombosis and the inflammation underlying diabetes.

In conclusion, this study suggests increased intracellular levels in and release of CD40L by platelets of diabetic subjects, and induction of CD40L expression and release from platelets by glucose and AGEs as a novel mechanism that could account for accelerated atherogenesis in diabetes.

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Conflict of interest

None declared.

References

- Schonbeck U, Libby P. CD40 signaling and plaque instability. *Circ Res* 2001;**89**:1092-103.
- Lutgens E, Daemen MJ. CD40-CD40L interactions in atherosclerosis. *Trends Cardiovasc Med* 2002;**12**:27-32.
- Mach F, Schonbeck U, Sukhova GK, Atkinson E, Libby P. Reduction of atherosclerosis in mice by inhibition of CD40 signalling. *Nature* 1998;**394**:200-03.
- Lutgens E, Gorelik L, Daemen MJ *et al*. Requirement for CD154 in the progression of atherosclerosis. *Nat Med* 1999;**5**:1313-16.
- Schonbeck U, Sukhova GK, Shimizu K, Mach F, Libby P. Inhibition of CD40 signaling limits evolution of established atherosclerosis in mice. *Proc Natl Acad Sci USA* 2000;**97**:7458-63.
- Lutgens E, Cleutjens KB, Heeneman S, Kotliansky VE, Burkly LC, Daemen MJ. Both early and delayed anti-CD40L antibody treatment induces a stable plaque phenotype. *Proc Natl Acad Sci USA* 2000;**97**:7464-9.
- Aukrust P, Muller F, Ueland T *et al*. Enhanced levels of soluble and membrane-bound CD40 ligand in patients with unstable angina. Possible reflection of T lymphocyte and platelet involvement in the pathogenesis of acute coronary syndromes. *Circulation* 1999;**100**:614-20.
- Schonbeck U, Varo N, Libby P, Buring J, Ridker PM. Soluble CD40L and cardiovascular risk in women. *Circulation* 2001;**104**:2266-8.
- Heeschen C, Dimmeler S, Hamm CW *et al*. Soluble CD40 ligand in acute coronary syndromes. *N Engl J Med* 2003;**348**:1104-11.
- Varo N, de Lemos JA, Libby P *et al*. Soluble CD40L: risk prediction after acute coronary syndromes. *Circulation* 2003;**108**:1049-52.
- Blake GJ, Ostfeld RJ, Yucel EK *et al*. Soluble CD40 ligand levels indicate lipid accumulation in carotid atheroma: an in vivo study with high-resolution MRI. *Arterioscler Thromb Vasc Biol* 2003;**23**:e11-14.
- Marx N, Imhof A, Froehlich J *et al*. Effect of rosiglitazone treatment on soluble CD40L in patients with type 2 diabetes and coronary artery disease. *Circulation* 2003;**107**:1954-7.
- Varo N, Vicent D, Libby P *et al*. Elevated plasma levels of the atherogenic mediator soluble CD40 ligand in diabetic patients: a novel target of thiazolidinediones. *Circulation* 2003;**107**:2664-9.
- Sheetz MJ, King GL. Molecular understanding of hyperglycemia's adverse effects for diabetic complications. *JAMA* 2002;**288**:2579-88.
- Vlassara H, Palace MR. Diabetes and advanced glycation endproducts. *J Intern Med* 2002;**251**:87-101.
- Bucciarelli LG, Wendt T, Rong L *et al*. RAGE is a multiligand receptor of the immunoglobulin superfamily: implications for homeostasis and chronic disease. *Cell Mol Life Sci* 2002;**59**:1117-28.
- Wendt T, Tanji N, Guo J *et al*. Glucose, glycation, and RAGE: implications for amplification of cellular dysfunction in diabetic nephropathy. *J Am Soc Nephrol* 2003;**14**:1383-95.
- Kunt T, Forst T, Harzer O *et al*. The influence of advanced glycation endproducts (AGE) on the expression of human endothelial adhesion molecules. *Exp Clin Endocrinol Diabetes* 1998;**106**:183-8.
- Falet H, Barkalow KL, Pivniouk VI, Barnes MJ, Geha RS, Hartwig JH. Roles of SLP-76, phosphoinositide 3-kinase, and gelsolin in the platelet shape changes initiated by the collagen receptor GPVI/FcR gamma-chain complex. *Blood* 2000;**96**:3786-92.
- Lecine P, Villeval JL, Vyas P, Swencki B, Xu Y, Shivdasani RA. Mice lacking transcription factor NF-E2 provide in vivo validation of the pro-platelet model of thrombocytopoiesis and show a platelet production defect that is intrinsic to megakaryocytes. *Blood* 1998;**92**:1608-16.
- Henn V, Slupsky JR, Grafe M *et al*. CD40 ligand on activated platelets triggers an inflammatory reaction of endothelial cells. *Nature* 1998;**391**:591-4.
- Wautier MP, Massin P, Guillausseau PJ *et al*. N(carboxymethyl)lysine as a biomarker for microvascular complications in type 2 diabetic patients. *Diabetes Metab* 2003;**29**:44-52.
- Andre P, Nannizzi-Alaimo L, Prasad SK, Phillips DR. Platelet-derived CD40L: the switch-hitting player of cardiovascular disease. *Circulation* 2002;**106**:896-9.
- Appay V, Rowland-Jones SL. RANTES: a versatile and controversial chemokine. *Trends Immunol* 2001;**22**:83-7.
- Kingsley K, Huff JL, Rust WL, Carroll K, Martinez AM, Fitchmun M, Plopper GE. ERK1/2 mediates PDGF-BB stimulated vascular smooth muscle cell proliferation and migration on laminin-5. *Biochem Biophys Res Commun* 2002;**293**:1000-06.