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Enhanced soluble CD40 ligand contributes to endothelial cell dysfunction in vitro and monocyte activation in patients with diabetes mellitus: effect of improved metabolic control

Abstract *Aims/hypothesis:* Inflammation plays a pathogenic role in the development of accelerated atherosclerosis in diabetes. Soluble CD40 ligand (sCD40L) is enhanced in diabetes; however, the molecular mechanisms linking sCD40L to accelerated atherosclerosis in diabetes are still unclear. We tested the hypothesis that sCD40L may be involved in the vascular complications in diabetes and exerts its effect by triggering inflammatory reactions on mononuclear and endothelial cells (ECs). *Methods:* We studied 70 patients, 40 with type 2 and 30 with type 1 diabetes, with a history or physical examination negative for cardiovascular disease, and 40 non-diabetic and 30 healthy subjects, matched with the type 2 and type 1 diabetic patients, respectively. Plasma and serum sCD40L, and plasma soluble intercellular adhesion molecule-1, soluble vascular cell adhesion molecule-1, E-selectin and monocyte chemo-attractant protein-1 (MCP-1) were measured. Adhesion molecules and MCP-1 release, the ability to repair an injury in ECs, and O_2^- generation in monocytes were analysed in vitro after stimulation with serum from patients or controls. *Results:* Type 2 and type 1 diabetic patients had significantly higher sCD40L levels than controls. Furthermore, high sCD40L was associated with in vitro adhesion molecules and MCP-1 release, impaired migration in ECs and enhanced O_2^- generation in monocytes. Improved metabolic control was associated with a reduction of plasma sCD40L by 37.5% in 12 type 1 diabetic patients. Furthermore, elevated sCD40L in diabetic patients was significantly correlated with HbA$_1$c levels. *Conclusions/interpretation:* Upregulation of sCD40L as a consequence of persistent hyperglycaemia in diabetic patients results in EC activation and monocyte recruitment to the arterial wall, possibly contributing to accelerated atherosclerosis development in diabetes.

Keywords Atherosclerosis · Diabetes mellitus · Endothelium · Inflammation · Soluble CD40 ligand

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Abbreviations EC: Endothelial cell · MCP-1: Monocyte chemo-attractant protein-1 · NF-κB: Nuclear factor-κB · NO: Nitric oxide · PKC: Protein kinase C · ROS: Reactive oxygen species · sCD40L: Soluble CD40 ligand · sE-selectin: Soluble E-selectin · sICAM-1: Soluble intercellular adhesion molecule-1 · sVCAM-1: Soluble vascular cell adhesion molecule-1 · TZD: Thiazolidinedione

Introduction

Diabetes mellitus is a major source of morbidity in developed countries and, among its comorbid conditions, atherosclerosis is perhaps the most important.

Diabetes is associated with a variety of metabolic abnormalities, the principal of which is hyperglycaemia. Interestingly, prospective data have suggested a link between a

marker for chronic average glucose levels (HbA$_1$c) and cardiovascular morbidity and mortality [1]. However, there is considerable controversy with respect to the precise mechanism by which hyperglycaemia may contribute to the rapid development of coronary artery disease in diabetes.

CD40 ligand (CD40L, also known as CD154), a transmembrane protein structurally related to TNF-α, was originally identified on CD4+ T cells, but it was recently found also on activated platelets [2]. Both membrane-bound and soluble forms of this ligand may interact with CD40, which is constitutively expressed on B cells, macrophages, endothelial cells (ECs), and vascular smooth muscle cells, resulting in various inflammatory responses [3]. Recently the presence of T cells expressing CD40L within atherosclerotic lesions in humans has been reported [3], and the ability of gene disruption or anti-CD40L antibody treatment to limit the evolution and the instability of atherosclerotic plaque has been described [4]. Increased soluble CD40L (sCD40L) has been observed in unstable angina [5] and hypercholesterolaemia [6], and it has been recently reported that circulating sCD40L has a strong independent prognostic value among apparently healthy individuals [7] and patients with acute coronary syndromes [8, 9].

Thus, it is now generally accepted that CD40L–CD40 interaction is an initial event in atherothrombosis, leading in turn to the activation of several pro-inflammatory mediators [10]. In fact, CD40L may promote the expression of vessel-remodelling metalloproteinases and induce pro-coagulant activity in vascular cells, and the absence of CD40L affects the stability of arterial thrombi and delays arterial occlusion in vivo [11]. Moreover, ligation of CD40 on ECs inhibits cell migration after injury by increasing production of reactive oxygen species (ROS) [12], and upregulates monocyte chemo-attractant protein-1 (MCP-1), soluble E-selectin (sE-selectin), soluble vascular cell adhesion molecule-1 (sVCAM-1) and soluble intercellular adhesion molecule-1 (sICAM-1) [2, 13], all molecules that have been associated with atherothrombosis by mediating the recruitment and adhesion of monocytes and lymphocytes to the injured vessel wall [14, 15].

Worth noting is the recent demonstration of enhanced sCD40L in diabetic patients [16, 17], and the ability of a short-term treatment with thiazolidinediones (TZDs) to reduce it [16, 17], as a consequence of the anti-inflammatory effects of TZD [17]. However, some points regarding the relationship between sCD40L and diabetes are still unclear. First of all, the relationship between enhanced sCD40L and endothelial and monocyte function in diabetes has not yet been investigated. Second, no studies have yet demonstrated whether long-term intensive glycaemic control, as achieved by conventional antidiabetic therapy, may also diminish circulating sCD40L.

Thus, in the present study, we set out to investigate the possible role of sCD40L in vascular dysfunction in diabetes. In particular, we tested the hypothesis that sCD40L exerts its detrimental effect by triggering a complex group of inflammatory reactions both in vascular ECs and in circulating monocytes/macrophages. Finally, we analysed the effect of intensive glycaemic control on sCD40L levels in a 5-year study on 12 type 1 diabetic patients.

Subjects and methods

Subjects

Seventy patients with diabetes mellitus were studied. Forty had type 2 diabetes and 30 had type 1 diabetes, defined in accordance with the criteria of the American Diabetes Association [18]. None of the 70 patients had a history or physical examination positive for evidence of micro- or macrovascular complications. Forty non-diabetic subjects and 30 healthy subjects, age-, gender- and risk factor-matched with the type 2 and type 1 patients, respectively, were also studied. The baseline characteristics of patients and control subjects are detailed in Table 1. All the patients had stably achieved the therapeutic goal for both hypertension and hypercholesterolaemia (defined according to the JNC VI and ATP III guidelines, respectively). The study was approved by local ethics review committees and reviewed by the IRB of the G. d'Annunzio University Foundation. Written informed consent was obtained from each patient.

Study design

In the first study, a cross-sectional comparison of baseline sCD40L, sICAM-1, sVCAM-1, sE-selectin and MCP-1 was performed between patients and control subjects.

To assess the potential influence of improved metabolic control on sCD40L generation, a second study was performed in 12 of the 30 patients with type 1 diabetes. At the time of the cross-sectional study, these 12 type 1 diabetic patients were in poor metabolic control (HbA$_1$c>8%) despite taking insulin for months. These patients were examined every 3 months over a 5-year period for clinical and blood glucose monitoring, and intensive insulin therapy was adjusted to achieve improved metabolic control. No patient experienced a hypoglycaemic reaction during the study period. Blood samples were obtained before and at the end of this intensive monitoring and treatment programme for determination of fasting glucose, HbA$_1$c levels and sCD40L biosynthesis.

Laboratory procedures

Blood samples were collected in a fasting condition and immediately centrifuged at 4°C. Fasting plasma glucose was measured by the glucose oxidase method. HbA1c concentrations were measured by HPLC (Diamat Analyzer; BioRad, Richmond, CA, USA). The normal range was 4.2–6.0%, with an interassay CV of 3%. All the patients had at least three determinations per year and three determinations in the last month of the 5-year treatment period, and the mean of these was used for statistical analysis.

Table 1 Baseline clinical features of type 1 and 2 diabetic patients and matched nondiabetic controls

	Type 2 (*n*=40)	Controls (*n*=40)	Type 1 (*n*=30)	Controls (*n*=30)
Sex (F/M)	22/18	22/18	15/15	15/15
Age (years)	65±10	67±8	20±3.3	21±2.8
BMI (kg/m^2)	31.4±6	30.2±4	24.2±4.5	22.4±3
Duration of diabetes (years)	11.3±10.8	0	13.7±3.7	0
Fasting blood glucose (mg/dl)	182.8±67.3	91.5±7	187.4±84.7	78.4±8
HbA$_1$c (%)	7.6±2.3	4.4±0.8	8.87±1.3	4.2±1.1
Diagnosis of hypercholesterolaemia (*n*)	10	9	0	0
LDL cholesterol at enlistment (mg/dl)	91±4	90±5	89±5	93±3
Smoking (*n*)	4	4	0	0
Hypertension (*n*)	28	27	0	0
Systolic and Diastolic blood pressure at enlistment (mm Hg)	122±4/76±6	124±3/79±3	117±5/71±4	119±4/72±3
Family history of cardiovascular disease (*n*)	12	11	0	0
Treatment (*n*) with:				
Angiotensin receptor blockers	8	9	0	0
ACE-inhibitors	20	19	0	0
β-blockers	1	1	0	0
α-blockers	5	4	0	0
Ca^{2+} channel blockers	7	8	0	0
Diuretics	12	10	0	0
Statins	10	9	0	0

Data are means±SD or absolute values

Enzyme immunoassays

Serum and plasma samples were collected from all patients after a 12-h fast, and immediately stored at −80°C until analysis. Specific enzyme immunoassays for sCD40L, sICAM-1, sVCAM-1 and sE-selectin (Bender Medsystems, Vienna, Austria), and MCP-1 (BioSource, Camarillo, CA, USA) were used in triplicate as previously described [14], to increase the data accuracy. At our laboratory, the intraassay and interassay CV values were <6%. Finally, all analyses were performed by an operator blinded to the patients' characteristics.

sCD40L-dependent EC activation

HUVECs were harvested from fresh human umbilical cord veins and cultured as previously described [19]. After 3 and 18 h in culture, sICAM-1, sVCAM-1, sE-selectin and MCP-1 generation was measured by ELISA according to previously described and validated methods [14].

EC migration

HUVECs were isolated and cultured, and cell migration was analysed according to previously described and validated methods [19]. Cell viability was evaluated by the MTS [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxy-phenyl)-2-(4-sulphophenyl)-2H-tetrazolium, inner salt] colorimetric assay (Promega Corp., Madison, WI, USA). Some experiments were repeated in the presence of synthetic sCD40L (10 ng/ml and 1–10 μg/ml) (Alexis Biochemicals Corporation, San Diego, CA, USA) and anti-human sCD40L blocking monoclonal antibody (10 and 50 μg/ml) (Alexis). Incubation with isotype-matched control immunoglobulin served as negative control. Assays were performed in triplicate and repeated at least twice.

sCD40L-dependent monocyte activation

The isolation and culture of peripheral monocytes from five healthy blood donors and the analysis of O_2^- generation was assessed as previously described [9, 14]. Synthetic sCD40L (10 ng/ml and 1–10 μg/ml), anti-human blocking monoclonal antibody for CD40L (10 μg/ml) (Alexis), or control mouse IgG$_1$ (50 μg/ml) were added to some cell cultures. Furthermore, in some experiments chelerythrine was also added to test the hypothesis that protein kinase C (PKC) plays an important role in vascular superoxide production in diabetes.

Statistical analysis

The data were analysed by non-parametric methods to avoid assumptions about the distribution of the measured variables. ANOVA was performed with the Kruskal–Wallis method. Subsequent pairwise comparisons were made with

the Mann–Whitney U-test. The differences between baseline and post-treatment values were analysed with the Wilcoxon signed rank test. Correlations between biochemical parameters were assessed by the Spearman test. The data are expressed as means±SD. Statistical significance was considered to be indicated by $p<0.05$. All calculations were performed using the computer program SPSS 11.0.1.

Results

Circulating levels of sCD40L

Plasma sCD40L was significantly higher in patients with type 2 diabetes than in matched non-diabetic subjects (9.2± 3.4 vs 1.53±0.45 ng/ml, means±SD, $n=40$; $p<0.003$; Fig. 1a). Thirty-nine (97.5%) of 40 patients had sCD40L in excess of 2 SD above the control mean (Fig. 1a). Plasma sCD40L was also increased in the group of type 1 diabetic patients as compared with their matched control subjects (8.36±2.59 vs
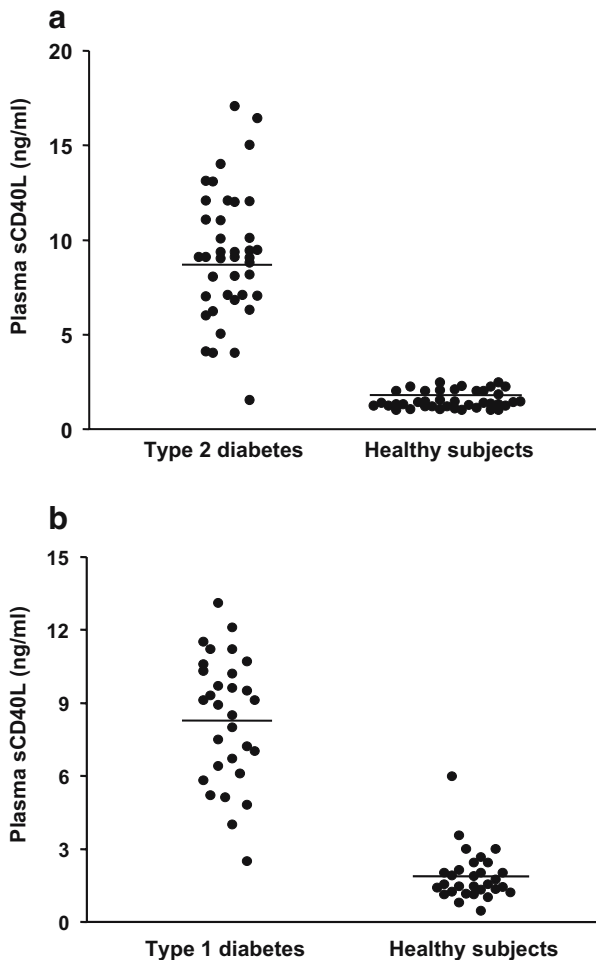

Fig. 1 Plasma levels of sCD40L in patients with type 2 diabetes ($n=40$) (**a**) and type 1 diabetes ($n=30$) (**b**) vs healthy subjects ($n=40$ and $n=30$, respectively). *Dots* represent individual measurements; *horizontal bars* represent mean values for each group. $p<0.0001$ (**a**); $p<0.0001$ (**b**)

1.87±1 ng/ml, means±SD, $n=30$; $p<0.003$; Fig. 1b). All but one (96.6%) of the 30 patients had sCD40L in excess of 2 SD above the control mean (Fig. 1b). Finally, no significant differences in plasma sCD40L were observed between patients with type 2 diabetes and patients with type 1 diabetes. Similar results were also observed when sCD40L was measured in serum (9.23±3.9 vs 1.92±0.8 and 9.3±2.6 vs 2± 1.1 ng/ml, respectively for type 2 and type 1 diabetes, means± SD $p<0.0001$). Thus, a significant correlation ($R^2=0.825$) was observed between plasma and serum sCD40L determinations. These data seem to suggest that, at least in diabetic patients, sCD40L measured in serum reflects circulating sCD40L rather than production of sCD40L from platelets activated during serum preparation.

In vivo biosynthesis of adhesion molecules and MCP-1 in diabetic patients

Endothelial perturbation was significantly higher in patients with diabetes (Fig. 2). In fact, both type 2 and type 1 diabetic patients showed higher ($p<0.0001$) levels of sICAM-1 (598±32 vs 367±29 and 511±18 vs 314±23 ng/ml, respectively), sVCAM-1 (1921±97 vs 943±64 and 1874±61 vs 1011±51 ng/ml, respectively) and, sE-selectin (61±6 vs 38±4 and 64±4 vs 37±3 ng/ml, respectively) as compared with control subjects (Fig. 2). Accordingly, both in type 2 and in type 1 diabetic patients we found a positive ($p<0.0001$) correlation between circulating sCD40L and circulating sICAM-1 ($R^2= 0.522$ and $R^2=0.492$), sVCAM-1 ($R^2=0.481$ and $R^2=0.536$), and sE-selectin ($R^2=0.512$ and $R^2=0.532$).

Similarly, MCP-1 levels were significantly ($p<0.0001$) higher both in type 2 and in type 1 diabetic patients with respect to controls (98±11 vs 57±6 and 87±8 vs 51±5 pg/ml, respectively), and a positive correlation ($p<0.0001$) was found between circulating sCD40L and circulating MCP-1 ($R^2=0.543$ and $R^2=0.421$).

Effect of sCD40L on endothelial activation in vitro

To further examine whether the higher grade of endothelial activation observed in diabetic patients was a direct consequence of enhanced circulating sCD40L, we next studied the effect of serum collected from diabetic patients on the generation of sICAM-1 and MCP-1 in ECs in vitro (Fig. 3). ECs spontaneously generated considerable levels of sICAM-1 and MCP-1 when cultured with serum collected from patients with both type 2 diabetes (623±72 vs 248±22 and 24.2±2 vs 10.3±2 ng/ml, respectively; Fig. 3; $p<0.0001$) and type 1 diabetes (711±77 vs 259±13 and 22±2 vs 9±1 ng/ ml, respectively; $p<0.0001$), whereas no significant generation was measured in cells cultured with serum from the controls (Fig. 3). The stronger stimulatory effect of sCD40L-rich serum from diabetic patients was replicated by exogenous sCD40L (10 ng/ml), while it was blocked by a blocking monoclonal antibody for CD40L (Fig. 3), thus confirming the causal role of sCD40L in sICAM-1 and
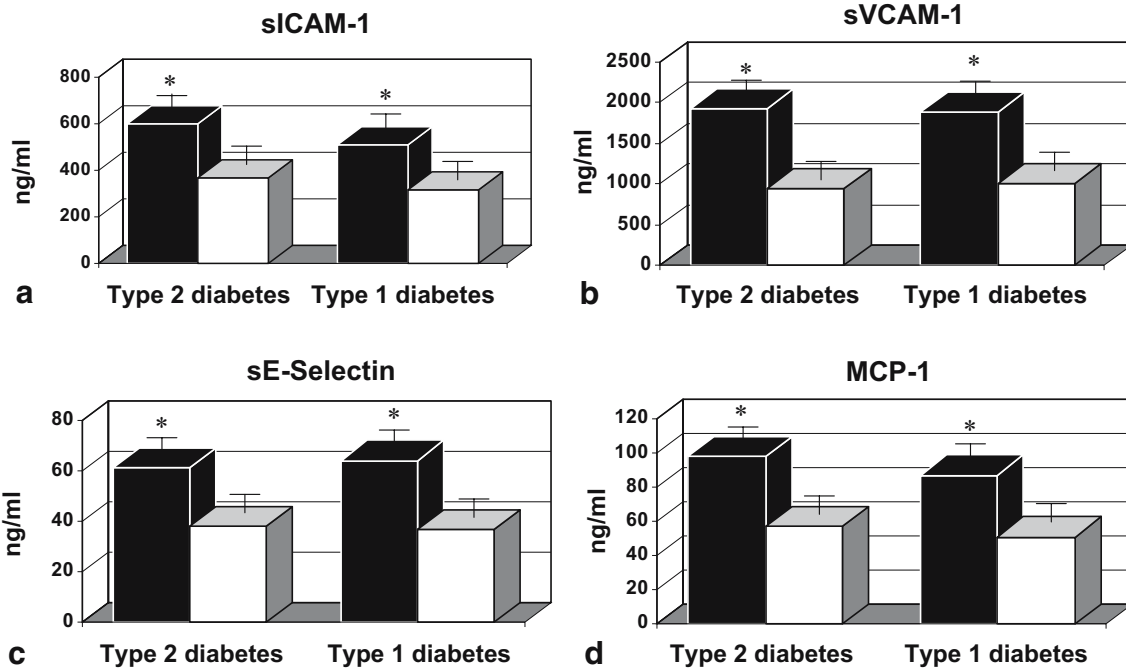
Fig. 2 Plasma levels of sICAM-1 (**a**), sVCAM-1 (**b**), sE-selectin (**c**) and MCP-1 (**d**) measured in patients with type 2 and type 1 diabetes (*black bar*) and in matched healthy controls (*open bar*). *Bars* and *vertical lines* represent means±SD. *$p<0.0001$ vs healthy subjects

MCP-1 generation in ECs. The high purity of recombinant sCD40L (>95%) and the low endotoxin content (<0.01 ng endotoxin/μg purified protein) excluded that the effect of recombinant sCD40L was due to contaminating endotoxin.

The incubation of cells with serum from healthy subjects containing anti-sCD40L monoclonal antibody excluded any non-specific effect of this antibody on cell function. Furthermore, the isotype-matched control antibody had no effect on sICAM-1 and MCP-1 generation.

Effect of sCD40L on EC migration in vitro

Migration (per cent of control, Fig. 4) was significantly ($p<0.001$) abrogated in ECs incubated for 3 or 18 h with serum from patients with type 2 diabetes and high sCD40L levels (−45.1±2.1 and −73.2±1.9%, respectively). In contrast, no significant inhibition was measured in ECs cultured with serum from matched control subjects with low sCD40L levels (−10±1.8 and −14±4.3%, respectively). Im-
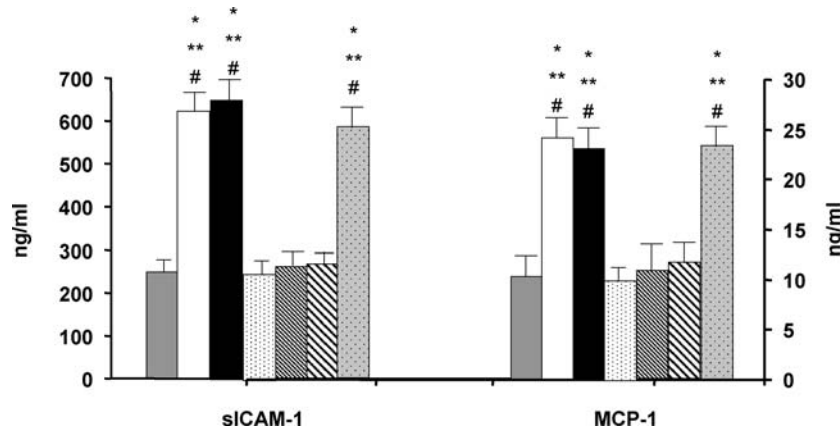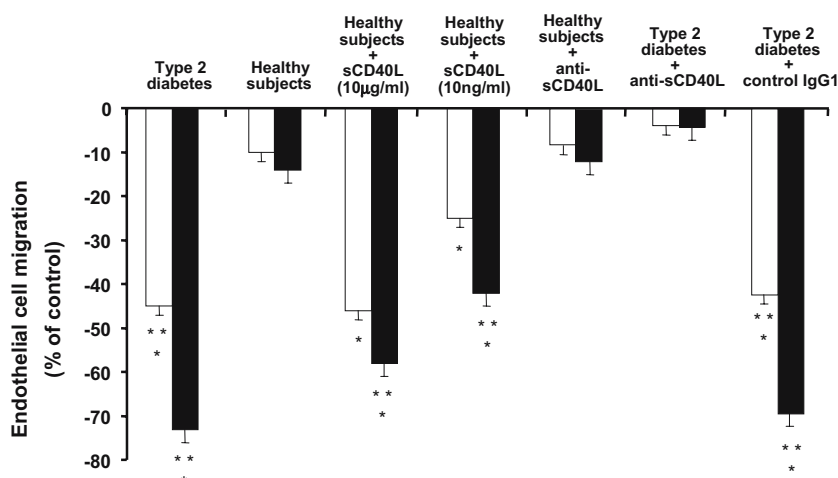

Fig. 3 Effect of serum from type 2 diabetic patients on spontaneous generation of sICAM-1 and MCP-1 in ECs after 3 h in culture. Cells were incubated with serum from healthy subjects (HS) (*grey bar*, *n*=40), type 2 diabetes (T2DM) (*open bar*, *n*=40), HS+exogenous sCD40L (*black bar*, *n*=40), HS+anti-sCD40L (*stippled bar*, *n*=40), HS+sCD40L+anti-sCD40L (*finely hatched bar*, *n*=40), T2DM plus anti-sCD40L (*coarsely hatched bar*, *n*=40), or T2DM plus isotype-matched control antibody (*stippled grey bar*, *n*=40). Note the inhibitory effect of blocking monoclonal antibody for CD40L. *Bars* and *vertical lines* represent means±SD. *$p<0.0001$ vs HS; **$p<0.0001$ vs HS+sCD40L+anti-sCD40L; #$p<0.0001$ vs T2DM+anti-sCD40L

Fig. 4 Effect of serum from type 2 diabetic patients on EC migration after 3 h (*open bar*) or 18 h (*black bar*) of incubation. Note the inhibitory effect of blocking monoclonal antibody for CD40L. *Bars* and *vertical lines* represent means±SD. *$p<0.001$ vs healthy subjects; **$p<0.001$ vs type 2 diabetes+anti-sCD40L

portantly, the stronger inhibitory effect of serum from diabetic patients was replicated by exogenous sCD40L (for 10 µg/ml: −46±2 and −58.3±3%, respectively, $p<0.001$; for 10 ng/ml: −25±1 and −42±2%, respectively, $p<0.001$), while it was completely reversed by neutralising antibody against CD40L (−4±2.4 and −4.3±3.2%, respectively). The anti-sCD40L monoclonal antibody had no effect on EC vitality as evaluated by the MTS colorimetric assay. Moreover, the incubation of ECs with serum from healthy subjects and anti-sCD40L monoclonal antibody excluded any non-specific effect of this antibody on cell function. Furthermore, no effect of isotype-matched control antibody on endothelial migration was found.

Notably, the antibody alone had no effect on EC migration (−1.2±2.8% after 18 h, p=NS vs medium alone), thus ruling out the hypothesis of any non-specific effect of the antibody per se. Similarly, the presence in culture medium of sCD40/anti-sCD40L complexes generated in antibody excess (1:5) did not significantly affect EC migratory ability (−1.0±1.9% after 18 h, p=NS vs medium alone), thus demonstrating that complexes of antibody and protein have direct effects on cell motility. Finally, the presence of the anti-oxidant *N*-acetyl-cysteine strongly antagonised the effect of sCD40L on EC migration, thus confirming the key role of oxidant stress in the pathophysiology of sCD40L-dependent endothelial dysfunction.

Effect of sCD40L on monocyte inflammatory status in vitro

To examine the relationship between circulating sCD40L and monocyte activity in diabetic patients, healthy monocytes were evaluated for spontaneous O_2^- generation after culturing with either serum from the 70 diabetic patients with broad-spectrum sCD40L levels (range 1.9–17.9 ng/ml), or serum from ten controls (<1.1 ng/ml). Monocytes spontaneously generated considerable levels of O_2^- when cultured with serum collected from both type 2 diabetic patients (11.3±2 nmol·60 min^{-1}·10^6 cells^{-1}, $p<0.0001$) and type 1 diabetic patients (12.3±1.9 nmol·60 min^{-1}·10^6 cells^{-1},

$p<0.0001$) (Fig. 5). In contrast, no detectable spontaneous O_2^- generation was observed in monocytes cultured with serum from controls (Fig. 5). The stronger stimulatory effect of serum from diabetic patients was replicated by exogenous sCD40L (also at plasma concentrations) (Fig. 5), while both were blocked by the blocking antibody for CD40L (Fig. 5) or chelerythrine, thus confirming the causal role of sCD40L and PKC in enhanced monocyte O_2^- generation. The anti-sCD40L monoclonal antibody had no effect on cell vitality as evaluated by the MTS colorimetric assay. Moreover, the incubation of cells with serum from healthy subjects containing anti-sCD40L monoclonal antibody excluded any non-specific effect of this antibody on cell function. The isotype-matched control antibody had no effect on
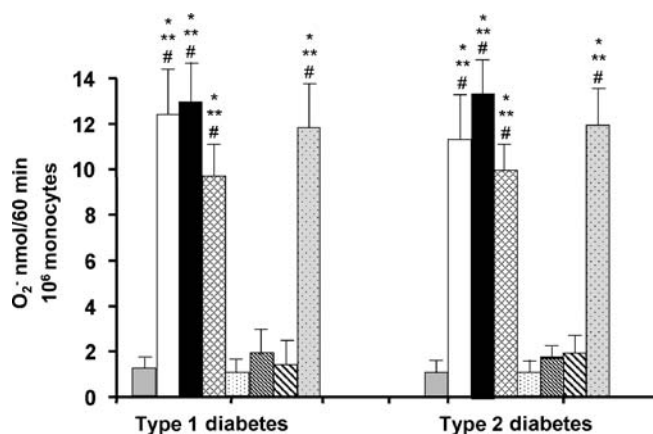

Fig. 5 Effect of serum from type 2 and type 1 diabetic patients on spontaneous generation of O_2^- in monocytes. Cells were incubated with serum from healthy subjects (HS) (*n*=10) (*grey bar*), diabetic patients (*open bar*, *n*=70), HS+exogenous sCD40L (10 µg/ml) (*black bar*, *n*=10), HS+exogenous sCD40L (10 ng/ml) (*cross-hatched bar*, *n*=10), HS+anti-sCD40L (*stippled bar*, *n*=10), HS+sCD40L (10 µg/ml)+anti-sCD40L (*finely hatched bar*, *n*=10), diabetic patients plus anti-sCD40L (*coarsely hatched bar*, *n*=70), or diabetic patients plus isotype-matched control antibody (*stippled grey bar*, *n*=70). Note the inhibitory effect of blocking monoclonal antibody for CD40L. *Bars* and *vertical lines* represent means±SD. *$p<0.0001$ vs HS; **$p<0.0001$ vs HS+sCD40L+anti-sCD40L; #$p<0.0001$ vs diabetes+anti-sCD40L

O_2^- generation. Accordingly, both in type 2 and in type 1 diabetic patients we found a positive ($p<0.0001$) correlation ($R^2=0.523$ and $R^2=0.456$) between circulating sCD40L and monocyte O_2^- generation in vitro.

Influence of metabolic control on sCD40L biosynthesis

Twelve patients with type 1 diabetes who had not achieved adequate metabolic control ($HbA_1c>8\%$) were subjected to intensive monitoring and treatment over 5 years, and blood samples were obtained before and after metabolic control. At the end of this period, HbA_1c fell from 8.87 ± 1.3 to $7.3\pm1.1\%$ ($p<0.05$). Improvement in metabolic control was associated with a statistically significant ($p<0.05$) 37.5% reduction in sCD40L biosynthesis (10.4 ± 1.5 vs 6.5 ± 1 ng/ml, means$\pm$SD) (Fig. 6).

Next, we assessed the association between plasma sCD40L and glycaemic control in the two groups of diabetic patients. sCD40L showed a positive correlation with HbA_1c ($p<0.0001$) and plasma fasting glucose ($p<0.001$), both in the 40 patients with type 2 diabetes ($R^2=0.678$ and $R^2=0.421$, respectively) and in the 30 patients with type 1 diabetes ($R^2=0.622$ and $R^2=0.454$, respectively).
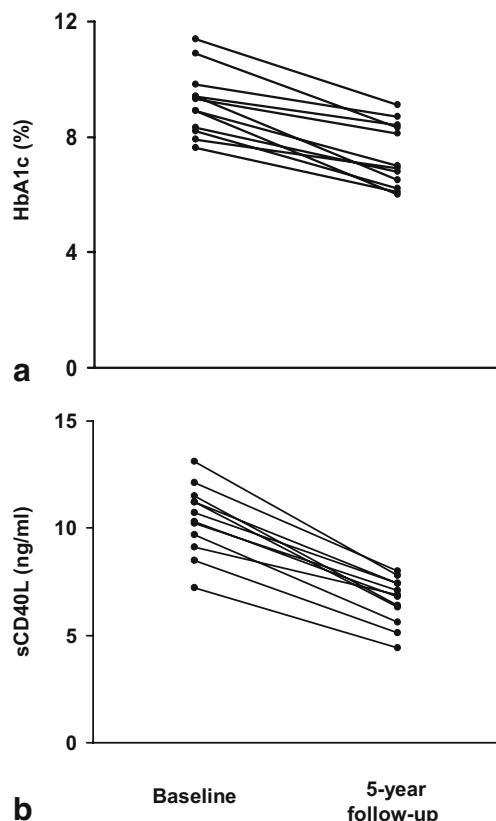

Fig. 6 Effect of improved glycaemic control on plasma sCD40L in 12 patients with type 1 diabetes and poor glycaemic control at baseline. $p<0.05$ (**a**); $p<0.05$ (**b**)

Discussion

Despite evidence of enhanced sCD40L recently reported in patients with diabetes [16, 17], no studies have yet addressed to what extent sCD40L may modulate the complex inflammatory reaction ultimately responsible for the accelerated atherosclerosis seen in diabetes, and whether long-term intensive glycaemic control might diminish circulating sCD40L and related inflammatory state.

Thus, this study: (1) provides in vitro evidence that enhanced sCD40L accumulation in diabetic plasma may modulate endothelial and monocyte function in humans; and (2) identifies a strong positive correlation between persistent hyperglycaemia and enhanced sCD40L generation in humans.

In this study, diabetic patients showed higher levels of sCD40L, significantly evident when compared with control subjects. In addition, the increase in sCD40L was irrespective of diabetes type, identifying in persistent hyperglycaemia the common pathogenetic element.

The striking effect of sCD40L in increasing the inflammatory reaction in ECs and monocytes raises the question of the origin of this mediator. Platelets are major contributors to enhanced sCD40L in patients with acute coronary syndrome [5] and hypercholesterolaemia [6], and studies on the cellular distribution of CD40L indicate that >95% of the circulating sCD40L exists in platelets [10]. It is important to note that we have previously described in patients with diabetes enhanced thromboxane A_2 biosynthesis [20, 21] and isoprostanes formation [21], mediators that may both induce and amplify platelet activation. Thus, platelets may be a source of localised high concentrations of pro-inflammatory CD40L, both on the surface and in the immediate environment as they shed sCD40L [10].

Activated platelets have been found to stimulate adhesion molecule and MCP-1 production in ECs through enhanced sCD40L secretion and direct platelet–endothelium contact mediated by CD40L expression on the platelet surface [2]. Such a mechanism has recently been found to be operative in patients with unstable angina [5], and it is conceivable that such a platelet–endothelium interaction may also contribute to the enhanced expression of adhesion molecules and MCP-1 observed in patients with diabetes. In fact, our observation that sCD40L is positively correlated with circulating adhesion molecules and MCP-1 in vivo, and may influence the expression of sICAM-1, sVCAM-1, sE-selectin and MCP-1 in ECs in vitro, strongly supports this hypothesis.

Importantly, because none of our patients received aspirin throughout the study, we cannot exclude a potential positive effect of aspirin therapy on sCD40L levels in diabetes. Thus, despite recent evidence [7] suggesting that aspirin administration did not alleviate the enhanced risk of future cardiovascular events associated with high sCD40L levels in healthy subjects, additional studies are required to investigate this point in patients with diabetes mellitus.

Alternatively, we cannot exclude that vascular smooth muscle cells, macrophages and lymphocytes may be involved also, at least in part, in sCD40L generation in the setting of diabetes. Thus, a contribution to enhanced sCD40L

in diabetic patients could be due to inflammatory cells, chronically activated in patients with diabetes.

Thus, our data contribute to the identification of sCD40L as the crucial link between a subclinical inflammatory state at baseline and vessel hyper-responsiveness to injury, ultimately responsible for the accelerated atherosclerosis seen in diabetes. In this light, enhanced sCD40L in diabetic patients is not simply a marker of higher immune activation, but is also actively involved in pathogenetic processes in these patients. Indeed, whatever the cellular source(s), sCD40L may both indirectly and directly have important pathophysiological consequences in patients with diabetes. In fact, CD40L–CD40 interaction may be involved in the process of atherothrombosis through the complex biological effects that it exerts on a number of cell types. First, sCD40L may exercise profound influences on endothelial function. In fact, we demonstrate in this study that sCD40L stimulates the endothelial expression of adhesion molecules critically involved in the early phase of atherogenesis, may activate ECs to release MCP-1, a major signal for inflammatory cell accumulation in the injured vessel [14], and finally exerts a profound inhibitory effect on endothelial migration, a process considered critical for the re-endothelialisation of the injured vessel. EC migration is a tightly regulated process modulated by a complex set of inward and outward signals leading to dynamic and coordinated changes in cytoskeleton organisation and cell adhesion. In particular, adequate endothelium-derived nitric oxide (NO) availability is required for EC migration. In this regard, Urbich et al. [12] have recently demonstrated that sCD40L inhibited EC motility in vitro by reducing NO through an increased generation of ROS. Indeed, the involvement of ROS in the anti-migratory effect of sCD40L has been demonstrated by using anti-oxidant treatments both in the study from Urbich et al. [12] and in our own.

In addition to the effects on ECs, sCD40L may exercise profound influences on monocytes also. In fact, in the present study we found that the raised sCD40L levels in serum from diabetic patients had enhancing effects on spontaneous ROS generation in monocytes. Thus, if this enhancement also occurs in vivo, it may be directly involved in the rapid progression of atherosclerosis. In fact, the generation of ROS has profound and wide-ranging effects that can dramatically increase vascular toxicity and initiate a cascade of molecular and cellular responses. Superoxide anion can inactivate NO, reducing the vasoactive levels of NO and diminishing NO-dependent EC migration [12]. Furthermore, these free radicals with strong cytotoxic potency may cause either direct arterial wall injury or may initiate and/or accelerate secondary processes, including activation of phagocyte–platelet–EC interactions [22], thus further sustaining the detrimental action of platelet-derived sCD40L on ECs. Enhanced O_2^- generation may also amplify inflammatory gene production. In fact, many vascular biologists now accord a central role to augmented transcription of several atherosclerosis-related genes by the oxidant-sensitive regulatory pathway involving nuclear factor κB (NF-κB) [23]. Exposure to extracellular O_2^- activates the NF-κB regulatory complex and triggers in ECs and monocytes the transcription of genes that encode certain growth factors, adhesion molecules, chemo-attractant cytokines such as MCP-1, and enzymes that can influence extracellular matrix metabolism. Thus, increased O_2^- generation in monocytes may enhance the synthesis of MCP-1 in these same cells through an autocrine mechanism [24], possibly representing a vicious circle implicated in accelerated atherosclerosis. Moreover, O_2^- generation might evoke a secondary adhesion molecule and MCP-1 response from ECs, and establish a positive, self-stimulatory paracrine feedback loop, amplifying and sustaining the proliferative response [23]. Finally, superoxide generation by activated platelets may aggravate endothelial function at the site of platelet aggregation, possibly contributing to acute vessel occlusion. It is also important to consider the fact that superoxide production in monocytes is due to increased NADPH oxidase activity, an enzyme which has been linked strongly in causing endothelial dysfunction in experimental models and in vessels from diabetic patients [25, 26]. Thus, our data seem to suggest that sCD40L, in addition to inducing direct inhibition of EC migration, is also involved in the control of the endothelial- and monocyte-mediated inflammatory reactions, ultimately responsible for accelerated atherosclerosis in diabetes.

The fact that impaired glycaemic control, rather than the attendant macrovascular complications, is responsible for enhanced formation of sCD40L in diabetes is supported in this study by the absence of manifest coronary, cerebrovascular and peripheral disease in studied type 2 and type 1 diabetic patients. In addition, the active role of sCD40L in modulating vascular reactivity has also been demonstrated in this study in vitro by the use of the CD40L-neutralising antibody.

We further examined the relationship between metabolic control and sCD40L formation by studying 12 type 1 diabetic patients with inadequate glycaemic control before and after intensive insulin treatment, and closer monitoring. Reduced blood glucose levels were associated with a fall in plasma sCD40L biosynthesis rates, the average extent of which showed a remarkably good fit with the linear relationship between blood HbA_1c and blood sCD40L, as established in the two groups of diabetic patients under baseline conditions. Improvement of metabolic control in these patients was accompanied by a statistically significant reduction in sCD40L biosynthesis by 37.5%. On the basis of these findings, it is tempting to speculate that changes in the rate of sCD40L biosynthesis may represent an important biochemical link between altered glycaemic control, platelet activation and vascular dysfunction in diabetes.

A potential limitation of this study is that we have analysed only the soluble but not the membrane-bound CD40L, despite the fact that cell-surface CD40 and CD40L expression are also relevant components of atherogenesis. However, it should be noted that at this time only sCD40L but not membrane-bound CD40L has been identified as an independent predictor of future cardiovascular events in humans [7, 8].

In conclusion, our results are consistent with the hypothesis that upregulation of sCD40L in patients with diabe-

tes results in the activation of ECs and in the recruitment of monocytes and tissue macrophages to the arterial wall, possibly contributing to the accelerated development of atherosclerosis seen in diabetes. Further understanding of the mechanism(s) by which sCD40L is produced and acts might provide insight into therapies to limit the progression of diabetic atherosclerosis.

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