CD40L induces inflammation and adipogenesis in adipose cells – a potential link between metabolic and cardiovascular disease

Anna Missiou1*, Dennis Wolf1*, Isabel Platzer1; Sandra Ernst1; Carina Walter1; Philipp Rudolf1; Katja Zirlik2; Natascha Köstlin1; Florian K. Willecke1; Christian Münkel1; Uwe Schönbek2; Peter Libby3; Christoph Bode4; Nerea Varo5*; Andreas Zirlik1*

1Department of Cardiology, University of Freiburg, Freiburg, Germany; 2Department of Hematology and Oncology, University of Freiburg, Freiburg, Germany; 3External Research and Development, Pfizer, New York, New York, USA; 4Donald W. Reynolds Cardiovascular Research Center, Department of Medicine, Brigham and Women’s Hospital and Harvard Medical School, Boston, Massachusetts, USA; 5Department of Clinical Chemistry, University of Navarra, Pamplona, Spain

Summary

CD40L figures prominently in atherosclerosis. Recent data demonstrate elevated levels of sCD40L in the serum of patients with the metabolic syndrome (MS). This study investigated the role of CD40L in pro-inflammatory gene expression and cellular differentiation in adipose tissue to obtain insight into mechanisms linking the MS with atherosclerosis. Human adipocytes and preadipocytes expressed CD40 but not CD40L. Stimulation with recombinant CD40L or membranes over-expressing CD40L induced a time- and dose-dependent expression of IL-6, MCP-1, IL-8, and PAI-1. Supernatants of CD40L-stimulated adipose cells activated endothelial cells, suggesting a systemic functional relevance of our findings. Neutralising antibodies against CD40L attenuated these effects substantially. Signalling studies revealed the involvement of mitogen-activated protein kinases and NFκB. Furthermore, stimulation with CD40L resulted in enhanced activation of C/EBPα and PPARγ and promoted adipogenesis of preadipose cells in the presence and absence of standard adipogenic conditions. Finally, patients suffering from the metabolic syndrome with high levels of sCD40L also displayed high levels of IL-6, in line with the concept that CD40L may induce the expression of inflammatory cytokines in vivo in this population. Our data reveal potent metabolic functions of CD40L aside from its known pivotal pro-inflammatory role within plaques. Our data suggest that CD40L may mediate risk at the interface of metabolic and atherothrombotic disease.

Keywords

CD40L, adipocytes, preadipocytes, inflammation, metabolic syndrome

Introduction

The CD40/CD40L dyad fulfills powerful functions in inflammation and immunity (1). Beyond its crucial role in adaptive immune response CD40L potently promotes inflammatory processes in atherogenesis (2, 3). Upon stimulation with CD40L cells typically resident in atherosclerotic plaques such as endothelial cells (EC), macrophages, and smooth muscle cells express various pro-inflammatory mediators, chemokines, adhesion molecules, procoagulants, and matrix degrading enzymes, generating a pro-atherogenic state (4). CD40L occurs in both membrane-bound and soluble form (sCD40L). Several reports demonstrated that sCD40L increases in patients with unstable angina compared to patients with stable angina or healthy volunteers and identifies those at risk within this collective (5–7). It also predicts the development of restenosis and independently correlates with traditional cardiovascular risk factors such as diabetes and hypercholesterolaemia (8, 9). Most recently, elevated levels of sCD40L were associated with diabetes and the metabolic syndrome (MS), raising the intriguing question whether CD40L may mediate some of the dramatically increased cardiovascular risk of this patient population (10–13).

The MS comprises a cluster of cardiovascular risk factors. One of its key components, obesity, predisposes for multiple chronic inflammatory diseases including atherosclerosis, diabetes mellitus, and cancer (14, 15). While for long misconceived as mere lipid storage organ, recent data identify adipose tissue as an active endocrine and immune organ. By production of inflammatory mediators such as tumour necrosis factor (TNF)α, interleukin (IL)-6, IL-8, monocyte chemoattractant protein (MCP)-1, and plasminogen activator inhibitor (PAI)-1 adipocytes and preadipocytes may directly promote atherogenesis (16–19). Interestingly, adipose tissue transforms in obesity, resulting in an enhanced expression of pro-inflammatory cytokines and an attenuated anti-inflammatory re-

*A.M. and D.W. contributed equally to this work. N.V. and A.Z. share senior authorship.

Correspondence to:
Andreas Zirlik, MD
Department of Cardiology, University of Freiburg
Breisacherstrasse 33, 79106 Freiburg, Germany
Tel.: +49 761 2707038, Fax: +49 761 2707006
E-mail: andreas.zirlik@uniklinik-freiburg.de

Received: July 29, 2009
Accepted after major revision: December 6, 2009
Prepublished online: February 19, 2010
doi:10.1160/TH09-07-0463
Thromb Haemost 2010; 103: 788–796
sponse. On histological phenotype, obese adipose tissue is characterized by an infiltration of inflammatory cells and an enlargement of adipose cells (20–22). The inflammatory environment activates the resident adipose cells and aggravates the inflammatory processes leading to a sustained systemic chronic inflammation which facilitates the development of atherosclerosis.

Although robust evidence implicates inflammation with MS and atherosclerosis, the underlying mechanisms linking obesity and atherosclerosis remain poorly understood. This study tested the hypothesis that CD40L initiates pro-inflammatory functions in adipose tissue which may contribute to the increased cardiovascular risk in metabolic patients.

**Methods**

**Cell isolation, culture, and stimulation**

Preadipocytes and adipocytes were isolated from human adipose tissue obtained from bariatric surgery and maintained in culture as described previously (23). All donors provided written informed consent. The protocol was approved by the Institutional Research Board of the University of Freiburg. All cells were starved at least 12 hours (h) prior to experiments. Cells were incubated with increasing concentrations of recombinant CD40L (1 μg/ml, 5 μg/ml, 10 μg/ml, Biotrend, Cologne, Germany) for indicated time points. Human umbilical vein endothelial cells (HUVEC) were obtained from PromoCell (Heidelberg, Germany).

**Isolation of membranes**

Fifty million fibroblasts from a murine cell line over-expressing human CD40L or respective control cells were solubilised with homogenisation buffer (20 mM HEPES pH 7.4, 1 mM EDTA, 250 mM saccharose) including protein inhibitors as described elsewhere (24). Cell lysates were centrifuged for 15 minutes (min) (4°C, 16,000 rpm) and obtained supernatant were centrifuged for 45 min (4°C, 30,000 rpm). The pellet was re-suspended in phosphate-buffered saline (PBS) and protein quantified colorimetrically (BCA, Pierce-Endogen, Boston, MA, USA).

**Enzyme-linked immuno-absorbent assay (ELISA)**

Human IL-6, IL-8, MCP-1, and PAI-1 were quantified in the supernatant of cultures using commercially available ELISA kits according to the instructions of the manufacturer (R&D and Techncolone, Minneapolis, MN, USA).

**Western blotting**

Cells were lysed, separated by SDS-PAGE under reducing conditions, and blotted to polyvinylidene difluoride membranes as described previously (25). Signalling studies were performed as described previously (26).

**Immunohistochemistry**

Immunohistochemistry was performed as described previously (27). Human preadipocytes were cultured on chamber slides, fixed in 4% paraformaldehyde, washed, and stained for CD40 and CD40L (Santa Cruz, Santa Cruz, CA, USA). After an overnight incubation with the respective primary antibody at 1:100, slides were washed, incubated with the corresponding secondary antibody for 2 h at 37°C and developed. Nuclear counterstaining was performed with hematoxylin.

**Reverse transcript PCR for CD40 and CD40L**

Reverse transcript PCR (RT-PCR) was performed as described previously (25). PCR was performed for 35 cycles with an annealing temperature of 59°C for CD40, 45°C for CD40L, and 63°C for GAPDH for 120 seconds (s), PCR employed the following primers: CD40, antisense, 5’-GGG ACC ACA GAC AAC ATC AG-3’, sense 5’-TGC CAG CCA GGA CAG AAA CT-3’; CD40L, antisense, 5’-CGG AAC TGT GGG TAT TT -3’, sense 5’-ACT TTT TGC TGT GCA TC -3’; GAPDH, antisense, 5’-ATG CAG CCC CGA ATG CTC CTC ATC GTG GCC ATC -3’, sense 5’-TTC TTT GAG GCC ATG TGG GCC AT-3’ (all obtained from MWG, Ebersberg, Germany).

**Adipose differentiation assays**

3T3-L1 cells were purchased from ATCC and differentiated in the presence or absence of a standard adipogenic cocktail (0.25 μM/ml dexamethasone, 0.5 mM/ml IBMX (isobutylmethylxanthine), 10 μg/ml insulin) and murine CD40L (5 μg/ml). After 10 days cells were stained for Oil-red-O as described previously (25). In parallel experiments total protein lysates from 3T3-L1 cells were taken after 0, 2, 4, 6, 8, 10, 14, and 20 days of differentiation and analysed for CD40 expression (Santa Cruz) by Western blotting. Similar differentiation experiments were also performed with primary human preadipocytes. For quantification of mRNA-expression, human preadipocytes were incubated with CD40L (10 μg/ml) for 3 and 7 days, total RNA was isolated by chloroform-phenol extraction and subjected to quantitative RT-PCR.
Quantitative RT-PCR

One μg RNA was transcribed into cDNA with use of the 1st strand cDNA synthesis kit (Roche, Mannheim, Germany). The cDNA obtained was subjected to quantitative real time-PCR with a Roche Light Cycler using the Light Cycler Fast Start DNA Master SYBR Green I kit (Roche). As endogenous control cyclophilin was employed. Primers for C/EBPα were F-primer 5'- AAG AAG TCG GTG AAC AAG AG -3', R-primer 5'- GCA GGC GTT CAT TGT CAC T -3', for PPARγ F-primer 5'- GAA ACT TCA AGA GTA CCA AAG TGC AA -3', R-primer 5'- AGG CTT ATT GTA GAG CTG AGT CTT CTC -3', and for cyclophilin F-primer 5'- CAT CTG CACT GCA GAC TGA G -3' and GAPDH R 5'- AGG GAA CAA GGA AAA CAT GGA A -3', and for Pref-1 F-primer 5'- GGA TGC TCA AAG GCT TGC GAG GAT GAC-3' , R-primer 5'- GCC CGA ACA TCT CTA GGA AAA CAT GGA A -3', and for Sox-9 F-primer 5'- AGG TGC TCA AAG GCT ACG ACT-3', R-Primer 5'- AGA TGT GCG TCT GCT CCG TG -3'. Conditions for all targets were: 45 cycles of 10 s at 95°C, 10 s at 58°C, 15 s at 72°C. The mRNA expression of C/EBPα, PPARγ, and Sox-9 was analysed employing the 2-ΔΔCt method (28).

Stimulation of HUVECs with the supernatants of adipose cells

Preadipocytes or adipocytes were stimulated in the presence or absence of CD40L (10 μg/ml) or TNFα (50 ng/ml) and a neutralising CD40L antibody (Santa Cruz, 10 μg/ml). Medium was changed after 2h and supernatants were collected after another 24 h. IL-6, IL-8, MCP-1, and PAI-1 were quantified by ELISA, cell lysates were analysed for ICAM-1. Alternatively, endothelial cells were detached and analysed for surface expression of ICAM-1 by flow cytometry.

Clinical study

A total of 234 obese and 144 non-obese patients attending the Cardiovascular Risk Area of the University Clinic of Navarra (Spain) for a general check-up were included in the study. Subjects were free from clinically apparent atherosclerotic disease based on absence of history of coronary disease, stroke, or peripheral artery disease and normal electrocardiogram. Exclusion criteria were impaired renal or liver function, arteritis, and connective tissue diseases. None of the subjects studied presented any inflammatory disease or condition associated with alterations in plasma sCD40L levels. The local committee on human research approved the study, performed in accordance with the Declaration of Helsinki, and all participants gave written informed consent. All participants underwent a complete medical examination and anthropometric measurements were taken. Blood pressure was measured using a mercury sphygmomanometer in a sitting position. The average of two measurements was considered. Subjects were classified as obese and non-obese according to body mass index (BMI) (obese ≥30 kg/m²).

Biochemical analysis

Serum and plasma were collected in Vacutainer® tubes. Fasting serum glucose, cholesterol, triglycerides (TG) and high-density lipoprotein (HDL)-C were measured by standard laboratory techniques. The HOMA-IR (Homeostasis Model Assessment of insulin resistance) was calculated as insulin (μU/ml)×glucose (mM)/22.5. Serum levels of sCD40L and IL-6 were measured by ELISA (R&D systems and R&D systems). The intra-assay variation was <10%. Inter-assay variation was <15%.

Data analysis

Western blots were analysed densitometrically using ImageJ (NIH software, National Institutes of Health, Bethesda, MD, USA). The statistical analysis was performed with SPSS (version 13.0; SPSS, Chicago, IL, USA). Statistics employed the Student's two-tailed t-test for paired or unpaired values (where appropriate) to assess statistical differences between experimental conditions. Data of at least three experiments were pooled and presented as mean ± standard error of the mean (SEM). P<0.05 was considered statistically significant. Normal distribution in the clinical study was tested with the Shapiro Wilks test.

Results

Adipocytes and preadipocytes express CD40 but not CD40 ligand

Various cell types express CD40L and its receptor CD40 (3). To investigate whether adipose tissue and its cellular fractions do we performed RT-PCR on respective RNA samples. While preadipocytes and adipocytes constitutively expressed CD40, no CD40L expression could be detected (Fig. 1A). Stimulation with 20 ng/ml TNFα for 24 h showed no induction of CD40L mRNA expression. Similar results were obtained by immunohistochemistry (Fig. 1B). Mature adipocytes (floaters) isolated from subcutaneous tissue of patients with or without obesity tended to negatively correlate with BMI (Pearson r=-0.58, p=0.226, N=6).
CD40L induces pro-inflammatory cytokine expression in adipocytes and preadipocytes

To test the hypothesis that CD40L promotes inflammation in adipose tissue we explored the regulation of the pro-inflammatory cytokine IL-6, the chemokines MCP-1 and IL-8, and the inhibitor of fibrinolysis PAI-1 by CD40L in supernatants from human preadipocytes and adipocytes. Stimulation of preadipocytes with CD40L (10 μg/ml) for 24 h significantly increased the constitutive expression of PAI-1, MCP-1, IL-8, and IL-6 from 56.2 ± 17.3 ng/ml, 0.5 ± 0.3 ng/ml, 0.2 ± 0.1 ng/ml, and 0.9 ± 0.5 ng/ml to 150.2 ± 39.1 ng/ml, 2.7 ± 1.0 ng/ml, 1.1 ± 0.3 ng/ml, and 5.2 ± 4.2 ng/ml, respectively.

Figure 1: CD40 but not CD40L is expressed by adipose cells. A) CD40 and CD40L mRNA expression on preadipocytes and adipocytes was measured by RT-PCR. Preadipocytes and adipocytes showed CD40 mRNA expression whereas no CD40L mRNA expression could be detected. B) Preadipocytes grown on cover slips were stained for CD40 and CD40L. Again preadipocytes express CD40 but not CD40L (representative images; N=4).

Figure 2: CD40L induces inflammatory cytokine expression in adipose cells. A) Preadipocytes stimulated with CD40L (10 μg/ml) released substantial amounts of PAI-1, IL-8, MCP-1, and IL-6 in a time-dependent fashion as assessed by ELISA. Results of six experiments performed in triplicate are presented as mean ± SEM. * indicates a p-value of ≤0.05. B) Upon stimulation of preadipocytes with CD40L, PAI-1, IL-8, MCP-1, and IL-6 accumulated in the supernatants dose-dependently as assessed by ELISA. Results of six experiments performed in triplicates are presented as mean of percent of control ± SEM. * indicates a p-value of ≤0.05. C) Membranes isolated from a CD40L-overexpressing cell line (■) enhanced expression of PAI-1 and MCP-1 in the supernatants of adipocytes in a concentration-dependent fashion compared to membranes isolated from the same cell line not expressing CD40L as quantified by ELISA (❍). Results are expressed as mean ± SEM of four different experiments.
spectively (N=6, p≤0.05). Similarly, TNFα (20 ng/ml) augmented the expression of these mediators in the supernatant to 112.6 ± 21.8 ng/ml, 6.0 ± 2.2 ng/ml, 5.4 ± 0.5 ng/ml, and 5.4 ± 2.7 ng/ml, respectively, whereas interferon (IFN)γ had no effect (data not shown). CD40L-mediated cytokine induction was time- and dose-dependent (► Fig. 2A and B). Similar results were obtained after stimulation of adipocytes with CD40L (10 μg/ml) for 24 h. Expression of PAI-1, MCP-1, and IL-8 significantly increased from 31.2 ± 18.7 ng/ml, 3.0 ± 2.1 ng/ml, and 1.2 ± 0.2 ng/ml to 92.8 ± 45.1 ng/ml, 28.9 ± 17.8 ng/ml, and 12.7 ± 10.2 ng/ml, respectively (N>5, p≤0.05). Since CD40L is expressed both in soluble form and membrane-bound in vivo, we sought to mimic cell-cell interactions: Membranes isolated from a murine cell line over-expressing human CD40L up-regulated pro-inflammatory cytokine production in differentiated human adipocytes compared to membranes isolated from respective control cells, corroborating the concept that CD40L induces inflammation in adipose tissue (Fig. 2C).

**CD40L activates classic pro-inflammatory signalling pathways**

CD40L activates a variety of signalling pathways in various cell types (3). Stimulation of preadipocytes with CD40L (10 μg/ml) resulted in activation of the mitogen-activated protein kinases Erk, p38, and JNK as well as of the transcription factor nuclear factor (NF)κB (► Fig. 3A and B), some of which have not only been implicated with inflammation but also with the development of obesity and its complications (29–31).

**CD40L induces adipogenesis involving C/EBPα and PPARγ**

Since fat mass and morphology represents a powerful predictor of cardiovascular risk, we further tested whether CD40L altered adipose cell differentiation (16, 20). Therefore, we studied the effect of CD40L (10 μg/ml) on adipogenesis in 3T3-L1 cells in the presence or absence of a standard adipogenic cocktail (IBMX, dexamethasone, insulin). Both under basal and stimulated conditions, CD40L markedly increased accumulation of fat droplets compared with respective controls as observed in bright field microscopy upon staining with Oil-red-O (► Fig. 4A). Similar results were obtained in primary human preadipocytes. Again, CD40L-stimulated cells showed a higher percentage of differentiated adipocytes demonstrating that CD40L indeed turns on adipogenesis (data not shown). Adipogenesis is driven by a transcriptional cascade including the master regulators C/EBPα and PPARγ (32). Incubation of human preadipocytes with CD40L (10 μg/ml) up-regulated the expression of CEBPα mRNA in a representative experiment after 3 and 7 days as well as the expression of PPARγmRNA after 7 days as assessed by quantitative RT-PCR (► Table 1). Whereas mRNA levels of the preadipocyte marker Pref-1 were not detectable, the transcription factor Sox-9 was up-regulated in CD40L-stimulated preadipocytes, most likely reflecting a remaining proportion of proliferating preadipocytes in in vitro differentiated adipocytes. Furthermore, CD40 expression markedly increased upon adipose differentiation as assessed by Western blotting (Fig. 4B).
Supernatants of CD40L-stimulated preadipocytes activate endothelial cells

To test whether the amount of cytokines released by adipose cells following stimulation with CD40L are capable of systemically activating atheroma-associated cell types, we challenged endothelial cells (EC) with supernatants of preadipocytes primed by CD40L. Upon co-incubation with supernatants of CD40L-stimulated preadipocytes, endothelial cells up-regulated the expression of IL-6, MCP-1, PAI-1, and IL-8 as assessed by ELISA (Fig. 5A). Since expression of adhesion molecules in endothelial cells is necessary for the recruitment of leukocytes to the sites of inflammatory lesions, we tested if the cytokines released by preadipocytes also results in increased expression levels of adhesion molecules, such as ICAM-1, VCAM-1 and selectins. Whereas supernatants of TNFα-primed preadipocytes induced a strong induction of ICAM-1 on endothelial cells, but not of VCAM-1 or selectins, CD40L only moderately enhanced ICAM-1 expression (Fig. 5B).

sCD40L and IL-6 levels in patients with the metabolic syndrome

To test for a potential link between sCD40L and IL-6 in vivo, 234 obese and 144 non-obese patients attending the Cardiovascular Risk Area of the University of Navarra were tested for both cytokines by ELISA. Table 2 shows the demographic and clinical characteristics of the study population. As expected obese patients presented significantly higher systolic and diastolic arterial blood pressure, BMI, cholesterol, low-density lipoprotein (LDL)-cholesterol, triglycerides, HOMA and glucose than non-obese patients. SCD40L levels were significantly higher in obese patients compared with non-obese (0.70 ± 0.07 vs. 0.86 ± 0.08ng/ml, respectively). CD40L significantly correlated with glucose levels and

<table>
<thead>
<tr>
<th>2-ΔΔCt</th>
<th>PPARγ</th>
<th>Control</th>
<th>CD40L</th>
</tr>
</thead>
<tbody>
<tr>
<td>3d</td>
<td>1.0 (0.7–1.4)</td>
<td>1.82 (1.39–2.36)</td>
<td></td>
</tr>
<tr>
<td>7d</td>
<td>1.0 (0.77–1.3)</td>
<td>0.98 (0.66–1.45)</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>2-ΔΔCt</th>
<th>C/EBPα</th>
<th>Control</th>
<th>CD40L</th>
</tr>
</thead>
<tbody>
<tr>
<td>3d</td>
<td>1.0 (0.85–1.18)</td>
<td>1.11 (0.74–1.68)</td>
<td></td>
</tr>
<tr>
<td>7d</td>
<td>1.0 (0.54–1.85)</td>
<td>1.23 (1.03–1.46)</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>2-ΔΔCt</th>
<th>Sox-9</th>
<th>Control</th>
<th>CD40L</th>
</tr>
</thead>
<tbody>
<tr>
<td>3d</td>
<td>1.0 (0.6–1.67)</td>
<td>1.74 (1.59–1.91)</td>
<td></td>
</tr>
<tr>
<td>7d</td>
<td>1.0 (0.87–1.15)</td>
<td>1.93 (1.64–2.28)</td>
<td></td>
</tr>
</tbody>
</table>
HOMA (r=0.11 and r=0.17, both p<0.05). No direct correlation was found between sCD40L and IL-6 levels or BMI. Patients were divided into two groups according to sCD40L levels above or below the mean. Interestingly, patients with sCD40L levels above the mean also showed significantly increased levels of IL-6 (Fig. 5C) in line with the notion that CD40L may relevantly up-regulate this mediator in vivo. Furthermore, given that adipose tissue expresses the receptor CD40 and that the circulating cytokine is biologically active, obese patients would be over-exposed to the detrimental effects of CD40L.

**Discussion**

Overwhelming evidence links obesity with chronic inflammation promoting metabolic disorders and cardiovascular complications (15, 18, 20, 22). In line with this concept, previous studies associated inflammatory states such as obesity, atherosclerosis, and the metabolic syndrome with elevated levels of pro-inflammatory mediators such as IL-6, IL-8, MCP-1, and PAI-1 (33). Although ample data suggest that inflammation is instrumental in the genesis of both metabolic and atherothrombotic disease, the mechanisms controlling the inflammatory response linking these two entities remain poorly understood. Given the dramatic epidemics of

![Figure 5: Supernatants of CD40L-stimulated preadipocytes activate endothelial cells and sCD40L plasma levels are associated with plasma levels of IL-6 in humans.](image)
metabolically-driven cardiovascular disease world-wide and their socio-economic magnitude increasing efforts are dedicated to explore the participating pathways. The present study identifies a pro-inflammatory role of the immune-modulatory molecule CD40L in adipose tissue which may contribute to the increased cardiovascular risk of metabolic patients.

We and others previously demonstrated that CD40L figures prominently in early and advanced atherosclerosis (4, 34–36). Here we show that CD40L induces a broad inflammatory response in both adipocytes and preadipocytes reflected in an increase of the pro-inflammatory cytokine IL-6, the chemokines MCP-1 and IL-8, and the inhibitor of fibrinolysis PAI-1. These data suggest that CD40L initiates systemic pro-inflammatory functions apart from its established role in plaque biology. Recombinant and membrane-bound CD40L were equally effective in and co-incubation with a CD40L-neutralising antibody attenuated inflammatory cytokine induction demonstrating specificity of our findings.

Recent studies associated elevated levels of sCD40L with the presence of the metabolic syndrome supporting our hypothesis that CD40L may play a role in metabolic disease (10, 37, 38). Basili et al. reported elevated sCD40L plasma concentrations in obese women compared to a normal weight control group suggesting that fat mass induces CD40L expression and contributes to sCD40L in blood (13). Similarly, Schernthaner et al. demonstrated elevated levels of sCD40L in morbidly obese patients which were significantly reduced after bariatric surgery (39). These observations are not in contrast to our finding that adipocytes and preadipocytes only express CD40L but not CD40L: Fat may not produce CD40L itself yet the inflammatory state that comes with obesity may attract cell types expressing CD40L (22, 40). Our data agree with a recent report by Poggi et al. suggesting that CD40L on T cells promotes inflammatory gene expression in adipose tissue (41). At the same time shedding of sCD40L from membrane-bound CD40L on inflammatory cells and platelets may increase plasma concentrations (42, 43), and thus directly stimulate adipose cells. Interestingly, Cipollone et al. recently demonstrated an association of high sCD40L levels in vivo with induction of adhesion molecules and MCP-1 in EC, impaired endothelial migration, and enhanced O2– generation in monocytes in vitro suggesting that sCD40L indeed functionally contributes to inflammation (44).

The mediators induced by CD40L in adipose cells in this study promote several pro-metabolic and pro-atherogenic functions: The chemokines IL-8 and MCP-1 contribute to the recruitment of macrophages and other immune cells into adipose tissue and the vessel wall (2, 45). Both also participate in atherogenesis in mice. MCP-1 further impairs insulin sensitivity and controls adipogenesis and adipocyte differentiation (46). IL-6 functions as a pro-inflammatory mediator activating inflammatory cells and promoting insulin resistance. PAI-1, highly expressed in inflamed adipose tissue, is an established risk factor for atherothrombotic diseases, including myocardial infarction (47). Consequently we hypothesised that CD40L-induced inflammation in adipose tissue may systemically contribute to pro-inflammatory, pro-atherogenic functions. Indeed, we demonstrate that media of CD40L-stimulated adipocytes or preadipocytes induce activation of endothelial cells, a cell type critical for plaque nascence and biology. In accord with this concept, our patients with the metabolic syndrome with high plasma levels of sCD40L also show high plasma levels of IL-6.

Previous studies showed that obesity is associated with adipogenesis and the formation of giant adipose cells (20, 21). In agreement with these findings we observe that CD40L stimulation induced lipid droplet accumulation and adipogenesis in 3T3-L1 cells. Furthermore obesity and weight gain strongly correlates with an increased cardiovascular risk. In contrast, weight loss reduces the risk for cardiovascular complications (48). Therefore our finding that CD40L induces adipogenesis might be another mechanism by which CD40L contributes to increased cardiovascular risk.

We present several lines of evidence that CD40L may mediate some of the cardiovascular risk in metabolic patients. Selective inhibition of CD40L may be a promising target for therapeutic intervention in metabolic disease and should be tested in in vivo models.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Non-obese</th>
<th>Obese</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, years</td>
<td>53 ± 1</td>
<td>56 ± 1</td>
</tr>
<tr>
<td>Male, %</td>
<td>66</td>
<td>87 *</td>
</tr>
<tr>
<td>Body mass index, kg/m²</td>
<td>24 ± 2</td>
<td>31 ± 2 *</td>
</tr>
<tr>
<td>Systolic arterial pressure, mmHg</td>
<td>125 ± 2</td>
<td>134 ± 1 *</td>
</tr>
<tr>
<td>Diastolic arterial pressure, mmHg</td>
<td>80 ± 1</td>
<td>85 ± 1 *</td>
</tr>
<tr>
<td>Glucose, mg/dl</td>
<td>99 ± 2</td>
<td>110 ± 3 *</td>
</tr>
<tr>
<td>HOMA</td>
<td>2.79 ± 0.18</td>
<td>4.92 ± 0.39 *</td>
</tr>
<tr>
<td>Total cholesterol, mg/dl</td>
<td>222 ± 3</td>
<td>235 ± 3 *</td>
</tr>
<tr>
<td>HDL-cholesterol, mg/dl</td>
<td>56 ± 1</td>
<td>48 ± 1 *</td>
</tr>
<tr>
<td>LDL-cholesterol, mg/dl</td>
<td>147 ± 3</td>
<td>160 ± 2 *</td>
</tr>
<tr>
<td>Triglycerides, mg/dl</td>
<td>96 ± 5</td>
<td>131 ± 5 *</td>
</tr>
</tbody>
</table>

**What is known about this topic?**
- CD40L is a marker and mediator of inflammatory disease such as atherosclerosis.
- Levels of sCD40L are elevated in patients with the metabolic syndrome.
- Mechanisms linking obesity and atherosclerosis remain poorly understood.

**What does this paper add?**
- CD40L induces the expression of inflammatory cytokines and promotes adipogenesis in human preadipocytes and adipocytes.
- Patients suffering from the metabolic syndrome with high levels of sCD40L also show high levels of IL-6.
- This may explain how CD40L mediates cardiovascular risk at the interface of metabolic and atherothrombotic disease.
Acknowledgement

This study was supported by grants from the Deutsche Forschungsgemeinschaft to Andreas Zirlik (ZI743/3–1 and 3–2) and a grant from the Bayer Science Foundation to Dennis Wolf.

References