C1q/TNF-related protein-1: an adipokine marking and promoting atherosclerosis

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Aims
We investigated the association of the adipokine C1q/TNF-related protein (CTRP) 1 with coronary artery disease (CAD), and the biological vascular effects of CTRP1.

Methods and results
We analysed CTRP1 levels in sera of CAD patients (n = 451) and non-CAD controls (n = 686), and in coronary endarterectomy specimens (n = 32), non-atherosclerotic internal mammary arteries (n = 26), aortic atherosclerotic plaques (n = 15), and non-atherosclerotic aortic samples (n = 10). C1q/TNF-related protein-levels were higher in sera, endarterectomy specimens, aortic atherosclerotic plaques, and peripheral blood mononuclear cells (PBMCs) from CAD patients compared with controls, and were related to CAD severity. The production of CTRP1 was profusely induced by inflammatory cytokines and itself caused a concentration-dependent expression of adhesion molecules and inflammatory markers in human endothelial cells, human peripheral blood monocytes, and THP-1 cells. C1q/TNF-related protein-1 induced p38-dependent monocyte-endothelium adhesion in vitro and the recruitment of leucocytes to mesenteric venules in C57BL/6 mice. Immunohistochemistry of atherosclerotic femoral arteries exhibited CD68 and VE-cadherin loci-associated increased CTRP1 expression in plaques. Compared with saline, intraperitoneal injection of recombinant CTRP1 protein (200 μg/kg) every other day promoted atherogenesis in apoE-/-/ mice at 24 weeks. However, pro-atherogenic effects were significantly attenuated in CTRP1-/-/apoE-/- double-knockout mice compared with apoE-/- mice, with a consistent decrease in vascular adhesion molecule, phospho-p38 and TNF-a expression and macrophage infiltration in plaque in CTRP1-/- and double-knockout mice. Tumour necrosis factor-a-induced expression of adhesion molecules and cytokines were lower in primary endothelial cells and macrophages from CTRP-/- mice than in those from C57BL/6 mice.

Conclusion
C1q/TNF-related protein-1 is a marker of atherosclerosis in humans and promotes atherogenesis in mice.

Keywords
CTRP1 • Adipokines • Coronary artery disease • Atherosclerosis • Endothelium

Translational perspective
We here conclusively demonstrate that the adipokine C1q/TNF-related protein (CTRP)-1 is associated in vivo with coronary artery disease, and has important pro-atherogenic effects in vitro and in vivo. Thus CTRP-1 is both a marker and a mediator, and may be a target for pharmacological interference.

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Introduction

In recent years, a family of adiponectin paralogues have been uncovered and designated as C1q/TNF-related proteins (CTRP). Members of the CTRP family play diverse roles in modulating the physiology, metabolism, and pathophysiology of the endocrine, immune, and cardiovascular systems. Some members of the CTRP family, such as CTRP1 and CTRP9, acting as disease antagonists, function in vitro to inhibit the pro-inflammatory pathway of lipopolysaccharide (LPS)-toll-like receptor (TLR) 4, and exert protective effects against myocardial injury in vivo. However, other members are related to the pathogenesis of cardiovascular diseases. One study has evidenced that CTRP1 levels are up-regulated in sera of hypertensive patients and it mediates angiotensin II-induced aldosterone production. In animal studies, LPS treatment causes tumour necrosis factor (TNF)-α- and interleukin (IL)-1β-mediated elevations of CTRP1 expression in the adipose tissue in db/db mouse and Zucker diabetic obese rat models. Recently, serum CTRP1 levels have been reported to be associated with coronary artery disease (CAD) in a small patient population, and with impairment of collateralization in such patients. However, extensive information on the relation of CTRP1 with atherosclerosis and underlying mechanisms is very limited.

We hypothesized that CTRP1 expression is increased in atherosclerosis, associates with vascular inflammation, and may itself be a promoter of vascular disease. In the present study, we thus evaluated serum CTRP1 concentrations in patients with angiographically documented CAD and non-CAD controls. We assessed CTRP1 protein levels in coronary endarterectomy specimens, non-atherosclerotic internal mammary arteries from patients undergoing bypass surgery, atherosclerotic plaques, and non-atherosclerotic aortic specimens, as well as in peripheral blood mononuclear cells (PBMCs) from patients with multi-vessel CAD and healthy volunteers. Using immunohistochemistry, we examined CTRP1 expression in atherosclerotic femoral artery specimens from patients with low-extremity artery occlusion and embolism. We also examined the in vitro production of CTRP1 in endothelial and monocyte cells treated with pro-inflammatory stimuli, such as TNF-α, IL-1β, and oxLDL. We used recombinant CTRP1 protein to stimulate human aortic endothelial cells (HAECs), human umbilical vein endothelial cells (HUVECs), human peripheral blood monocytes, and THP-1 cells, and analysed the induced expression of adhesion molecules and inflammatory cytokines. We carried out functional tests, including in vitro monocytoid cell-endothelium adhesion assays and in vivo intravital microscopy of mesenteric venules, to assess leucocyte-endothelium interactions and pro-inflammatory effects of CTRP1. Moreover, we performed intraperitoneal injection of recombinant CTRP1 to ascertain the atherogenic effect of CTRP1 in apoE−/− mice. We further explored atherogenic and pro-inflammatory effects of CTRP1 in CTRP1−/−/apoE−/− double-knockout mice and in knockout mouse-derived primary endothelial cells and macrophages. We here report on important associations of CTRP1 with vascular disease, with a demonstration of a causal role.

Methods

Participants

The in vivo human study included 451 consecutive CAD patients with stable angina (SA) or stable asymptomatic coronary heart disease (CHD) undergoing a coronary angiographic examination and interventional treatment from January to September 2010 at the Department of Cardiology, Rui Jin Hospital, Shanghai Jiaotong University School of Medicine, Shanghai. Six hundred and eighty-six subjects without evidence or history of vascular disease served as controls. Such control subjects were from outpatient clinics around our hospital, and receiving an annual physical check-up. They had normal resting electrocardiogram and exercise stress test, as well as normal carotid artery ultrasound examination and echocardiography. None had any evidence of cardiovascular disease (including exclusion of any past history of angina/myocardial infarction). Details of the study population are given in Supplementary material online.

The study protocol was approved by the Hospital Ethics Committee, and written informed consent was obtained from all patients. Since the study did not involve any intervention, it was not prospectively registered.

Coronary angiography

See Supplementary material online.

Biochemical measurements

We collected blood samples in patients after an overnight fasting. We stored all samples at −80°C until analysis. We determined serum CTRP1 levels using a commercially available ELISA kit (Biovendor R&D, Brno, Czech Republic). We assayed TNF-α and high-sensitivity C-reactive protein (hsCRP) levels by ELISA (R&D Systems, Minneapolis, MN, USA, and Biocheck Laboratories, Toledo, OH, USA, respectively). (See Supplementary material online.)

Materials

See Supplementary material online.

Cell cultures and experimental in vitro protocols

See Supplementary material online.

Human tissue sample management

We obtained coronary endarterectomy tissue specimens from 32 patients with angiographically documented severe multi-vessel CAD and undergoing coronary artery bypass graft (CABG). We also obtained surplus—otherwise discarded—segments of non-atherosclerotic internal mammary arteries from patients undergoing CABG surgery (n = 26). To minimize tissue-level variability on CTRP1 levels, we collected aortic atherosclerotic specimens from patients undergoing CABG (n = 15) and normal/non-atherosclerotic aortic samples from patients receiving an operation because of a thoracic aortic aneurysm, but without atherosclerosis (n = 10). We documented atherosclerotic lesions and the non-atherosclerosis status of aortas by CT angiography. For details, see Supplementary material online.

Isolation of peripheral blood monocytes

See Supplementary material online.
Animal studies
We conducted all animal experiments in accordance with experimental protocols approved by the Committee on Animal Resources of Shanghai Jiaotong University. We fed male apoE^{-/-} mice (purchased from the Beijing Laboratory Animal Research Center, Beijing, China) and male CTRP1^{-/-}/apoE^{-/-} mice (constructed by Shanghai Biomodel Organism, Shanghai, China), aged 8–10 weeks, a high-fat diet (21% fat, 0.2% cholesterol, 23% protein, and 40.5% carbohydrate) obtained from Shanghai SLAC Laboratory Animal Co., Shanghai, China. We maintained animals under pathogen-free conditions in the Animal Experiment Center of Rui Jin Hospital, Shanghai Jiaotong University School of Medicine. For CTRP1 treatment experiments, apoE^{-/-} mice received intraperitoneal injections of recombinant mouse CTRP1 protein (200 μg/kg, ~5 μg per mouse, n = 12) or saline (n = 12) every other day for 24 weeks. For experiments to explore the effects of CTRP1 deletion, we fed apoE^{-/-} (n = 12) and CTRP1^{-/-}/apoE^{-/-} mice (n = 12) a high-fat diet for 24 weeks. We then sacrificed mice and subjected them to analysis of lesion area in the aorta and to other analyses as detailed below. We obtained serum samples after mice had fasted for 4 h. We analysed levels of total cholesterol, HDL-C, LDL-C, triglycerides, and glucose, as described previously.12 We determined mouse serum TNF-α levels using murine Quantikine ELISA kits (R&D Systems), and mouse serum CTRP1 levels by the Biovendor (Biovendor R&D, Brno, Czech Republic) ELISA system.

We then quantified atherosclerotic lesion size by computerized image analysis after staining the entire aorta with Oil Red-O. We calculated the percentage of lesion coverage dividing the stained area by the total thoraco-abdominal aortic surface.13 We analysed the expression of adhesion molecules and other inflammatory factors in the aorta by Western blot and real-time PCR.

Generation of C1q/TNF-related protein-1 knockout mice
See Supplementary material online.

Western blotting
See Supplementary material online.

Quantitative real-time polymerase chain reaction analysis
See Supplementary material online.

Immunohistochemistry
See Supplementary material online.

Isolation and culture of mouse aortic endothelial cells
See Supplementary material online.

Assays of monocytoid cell adhesion to endothelial cells
See Supplementary material online.

Intravital microscopy
See Supplementary material online.

Statistical analysis
We here present continuous variables as mean and standard deviation (SD) or standard error of the mean (SEM), as specified, and summarize categorical data as frequencies or percentages. For categorical clinical variables, we evaluated differences between groups with the χ² test followed by Bonferroni’s correction to account for multiple comparisons. For continuous variables, we evaluated the existence of a normal distribution with the Kolmogrov–Smirnov test, and applied logarithmic or square-root transformations (for serum CTRP1 and TNF-α concentrations) on continuous variables showing a non-normal distribution. We analysed differences among patient groups by one-way analysis of variance (ANOVA) or the Kruskal–Wallis analysis, followed by post hoc analysis with the Bonferroni’s correction. We determined correlation between variables by the Pearson or Spearman correlation tests, as appropriate. We constructed two models for multivariable logistic regression to assess the independent determinants of CAD. In Model 1, we included traditional risk factors. In Model 2, we additionally adjusted the analysis for the adipokine CTRP1. We performed receiver operator characteristic (ROC) analysis of risk factors and biohumoral measurements with and without CTRP1. We used the C statistic to analyse the discriminatory capacity of Models 1 and 2. We also performed risk reclassification with the inclusion of CTRP1 by the method of Pencina et al., and determined the net reclassification improvement (NRI) accordingly.14,15 We checked model calibration using the Hosmer–Lemeshow χ² test. For cell experiments, data represent the average of six experiments. For animal experiments, we performed ANOVA and post hoc analysis (Bonferroni’s method) to assess the significance of changes compared with controls. We performed the Mann–Whitney U test for comparisons of two groups in multiple testing. All analyses used two-sided tests with an overall significance level (α) of 0.05, and were performed with the SPSS 15.0 for Windows (SPSS, Inc., Chicago, IL, USA) and SAS Version 9.1 (SAS Institute, Cary, NC, USA) software.

Results

Baseline characteristics of the study population
Table 1 illustrates the baseline characteristics and distribution of cardiovascular risk factors of the study populations. There were more males, a higher age, a higher percentage of hypertension and type 2 diabetes mellitus, higher lipoprotein (a) and triglyceride levels, as well as lower HDL-cholesterol and apoA-1, in the CAD group compared with controls. Notably, no significant difference was found in body mass index (BMI) between patient and control groups.

C1q/TNF-related protein-1 levels are higher in serum and peripheral blood mononuclear cells of coronary artery disease patients compared with controls, and are higher in atherosclerotic tissues than in the non-atherosclerotic arterial tissues
Serum levels of CTRP1, TNF-α, and hsCRP were significantly higher in CAD patients than in controls (for both comparisons, P < 0.001, Table 1). We further categorized the CAD patients in one-vessel and multi-vessel disease subgroups. There were higher CTRP1 levels in the multi-vessel disease subgroup (232.3 ± 41.1 ng/mL) than in the one-vessel disease subgroup (217.7 ± 39.6 ng/mL, P < 0.001). C1q/TNF-related protein-1 levels were related to the number of diseased coronary arteries (Spearman’s ρ = 0.223, P < 0.001), the atherosclerosis extent index (Pearson’s ρ = 0.206,
variable analyses, including traditional cardiovascular risk factors and biohumoral variables. To assess independent determinants of CAD, we performed multivariable analysis in Model 2, CTRP1 levels remained independently associated with CAD besides smoking, hypertension, diabetes, hyperlipidaemia, low apoprotein A, lipoprotein (a), and hsCRP were associated with CAD. When we included CTRP1 in the multivariable analysis in Model 2, CTRP1 levels remained independently associated with CAD besides the other conventional factors included in Model 1 (Table 2). Addition of CTRP1 marginally improved risk prediction (C-statistic, from 0.765 to 0.788, P = 0.016; NRI, 5.6%, P = 0.018). The calibration of Model 1 (0.711) and 2 (0.253) was good. Inclusion of CTRP1 resulted in a significant 4.9% improvement (Nagelkerke R² of Model 1 [0.266] and 2 [0.315], P < 0.001) in explaining the variations in the dependent variable.

### C1q/TNF-related protein-1 contribution to the prediction of coronary artery disease is additive to that of traditional risk factors and biohumoral variables

To assess independent determinants of CAD, we performed multivariable analyses, including traditional cardiovascular risk factors and biomeasurements reported in Table 1. In Model 1, male gender, age, smoking, hypertension, diabetes, hyperlipidaemia, low apoprotein A, lipoprotein (a), and hsCRP were associated with CAD. When we included CTRP1 in the multivariable analysis in Model 2, CTRP1 levels remained independently associated with CAD besides the other conventional factors included in Model 1 (Table 2). Addition of CTRP1 marginally improved risk prediction (C-statistic, from 0.765 to 0.788, P = 0.016; NRI, 5.6%, P = 0.018). The calibration of Model 1 (0.711) and 2 (0.253) was good. Inclusion of CTRP1 resulted in a significant 4.9% improvement (Nagelkerke R² of Model 1 [0.266] and 2 [0.315], P < 0.001) in explaining the variations in the dependent variable.

### C1q/TNF-related protein-1 is induced by inflammatory stimuli in endothelial and monocyctic cells, and itself induces inflammatory changes in these cell types

To probe whether inflammatory cytokines and oxLDL induce CTRP1 production, we stimulated HUVECs and THP-1 cells with TNF-α, IL-1β, and oxLDL. In both cell types, recombinant TNF-α, IL-1β, and oxLDL concentration dependently induced a significant increase of CTRP1 release in culture medium, with consistent increase of CTRP1 expression in cell lysates (see Supplementary material online, Figures S2–S7). To ascertain which cell types express CTRP1, we sorted out CD64⁺/CD16⁺ monocytes and

<table>
<thead>
<tr>
<th>Variable</th>
<th>Control group (n = 686)</th>
<th>Stable CAD group (n = 451)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Male/female</td>
<td>418/268</td>
<td>323/128</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Age (years)</td>
<td>63.2 ± 11.7</td>
<td>67.5 ± 9.7</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>24.9 ± 2.6</td>
<td>25.0 ± 3.2</td>
<td>0.624</td>
</tr>
<tr>
<td>Smoking (n, %)</td>
<td>272 (39.7)</td>
<td>208 (46.1)</td>
<td>0.01</td>
</tr>
<tr>
<td>Hypertension (n, %)</td>
<td>423 (61.9)</td>
<td>294 (65.2)</td>
<td>0.252</td>
</tr>
<tr>
<td>Systolic BP (mmHg)</td>
<td>132 ± 16</td>
<td>129 ± 19</td>
<td>0.537</td>
</tr>
<tr>
<td>Diastolic BP (mmHg)</td>
<td>79 ± 10</td>
<td>78 ± 11</td>
<td>0.786</td>
</tr>
<tr>
<td>Dyslipidaemia (n, %)</td>
<td>295 (43.0)</td>
<td>295 (65.4)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Type 2 diabetes mellitus (n, %)</td>
<td>199 (29.0)</td>
<td>144 (31.9)</td>
<td>0.192</td>
</tr>
<tr>
<td>Total cholesterol (mg/dL)</td>
<td>157.6 ± 38.2</td>
<td>164.9 ± 63.5</td>
<td>0.131</td>
</tr>
<tr>
<td>HDL-cholesterol (mg/dL)</td>
<td>48.6 ± 20.8</td>
<td>43.0 ± 17.8</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>LDL-cholesterol (mg/dL)</td>
<td>95.5 ± 31.2</td>
<td>96.8 ± 34.4</td>
<td>0.507</td>
</tr>
<tr>
<td>Triglycerides (mg/dL)</td>
<td>141.3 ± 84.1</td>
<td>156.8 ± 93.9</td>
<td>0.007</td>
</tr>
<tr>
<td>Apoprotein A (mg/dL)</td>
<td>132.8 ± 13.7</td>
<td>124.3 ± 28.1</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Apoprotein B (mg/dL)</td>
<td>86.8 ± 32.4</td>
<td>90.2 ± 26.1</td>
<td>0.257</td>
</tr>
<tr>
<td>Lipoprotein(a) (mg/dL)</td>
<td>14.7 ± 19.3</td>
<td>23.2 ± 21.5</td>
<td>0.004</td>
</tr>
<tr>
<td>Glucose (mg/dL)</td>
<td>96.7 ± 23.2</td>
<td>101.8 ± 28.8</td>
<td>0.007</td>
</tr>
<tr>
<td>Serum urea nitrogen (mg/dL)</td>
<td>14.9 ± 4.5</td>
<td>15.1 ± 4.6</td>
<td>0.241</td>
</tr>
<tr>
<td>Creatinine (mg/dL)</td>
<td>1.12 ± 0.33</td>
<td>1.21 ± 0.25</td>
<td>0.002</td>
</tr>
<tr>
<td>Uric acid (mg/dL)</td>
<td>3.69 ± 0.82</td>
<td>3.87 ± 1.03</td>
<td>0.012</td>
</tr>
<tr>
<td>CTRP1 (ng/mL)</td>
<td>185.4 ± 35.1</td>
<td>229.6 ± 42.5</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>hsCRP (mg/L)</td>
<td>5.12 ± 2.08</td>
<td>7.63 ± 4.38</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>TNF-α (pg/mL)</td>
<td>51.3 ± 31.0</td>
<td>62.9 ± 37.7</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

Data are mean ± SD or number (%).

CAD, coronary artery disease; HDL, high-density lipoprotein; LDL, low-density lipoprotein.

P < 0.001, and hsCRP level as an index of inflammation (Pearson’s r = 0.134, P < 0.001).
CD64+/CD16+ leucocytes from peripheral blood of CAD patients (n = 22) and controls (n = 18) to analyse CTRP1 expression by real-time RT-PCR. Of note, the mRNA level of CTRP1 was significantly increased in CD64+/CD16+ monocytes of CAD patients compared with those of healthy controls (P = 0.011) (see Supplementary material online, Figure S8).

We next investigated the effects of CTRP1 on HAECs, HUVECs, human peripheral blood monocytes, and THP-1 cells. In these cell experiments, we chose concentrations of CTRP1 based on serum levels, and also considering that tissue concentrations of this adipokine are higher than serum concentrations. After 24 h stimulation with CTRP1, protein and mRNA levels of adhesion molecules [vascular cell adhesion protein-1 (VCAM-1), intercellular adhesion molecule-1 (ICAM-1), and E-selectin] were significantly increased in a concentration-dependent manner in HAECs and HUVECs (Figure 2A, C–H and Supplementary material online, Figures S9). Likewise, an enhanced production of TNF-α and monocyte chemoattractant protein-1 (MCP-1) was induced by CTRP1 in the cytosol and culture medium of HAECs, HUVECs, human monocytes, and THP-1 cells (Figure 2A, B and I–L, and Supplementary material online, Figures S10 and S11). However, boiled CTRP1 did not show any of the above-mentioned cellular effects, indicating that these were not due to contaminated endotoxin in CTRP1 preparation (data not shown). Further experiments revealed that CTRP1 stimulation activated the p38 mitogen-activated protein kinase (MAPK) pathway with concurrent phosphorylation of the p65 component of nuclear factor (NF)-κB in HAECs, but not the ERK or the JNK pathways. Consistently, administration of the p38 inhibitor SB

Table 2 Multivariable regression analysis of independent risk factors for coronary artery disease

<table>
<thead>
<tr>
<th>Variables</th>
<th>OR (95% CI)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Model 1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>1.95 (1.32–2.88)</td>
<td>0.001</td>
</tr>
<tr>
<td>Age (per SD)</td>
<td>1.94 (1.62–2.31)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Body mass index (per SD)</td>
<td>1.26 (1.07–1.48)</td>
<td>0.005</td>
</tr>
<tr>
<td>Smoking</td>
<td>1.95 (1.39–2.73)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Hypertension</td>
<td>1.89 (1.35–2.64)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Diabetes</td>
<td>1.42 (1.03–1.94)</td>
<td>0.030</td>
</tr>
<tr>
<td>Hyperlipidaemia</td>
<td>1.66 (1.17–2.34)</td>
<td>0.004</td>
</tr>
<tr>
<td>Apoprotein A (per SD)</td>
<td>0.79 (0.66–0.94)</td>
<td>0.006</td>
</tr>
<tr>
<td>Lipoprotein (a) (per SD)</td>
<td>1.57 (1.33–1.84)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>hsCRP (per SD)</td>
<td>1.42 (1.22–1.66)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Model 2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>2.03 (1.36–3.03)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Age (per SD)</td>
<td>1.72 (1.43–2.06)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Body mass index (per SD)</td>
<td>1.24 (1.05–1.46)</td>
<td>0.012</td>
</tr>
<tr>
<td>Smoking</td>
<td>2.11 (1.49–2.97)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Hypertension</td>
<td>1.97 (1.40–2.78)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Diabetes</td>
<td>1.50 (1.09–2.07)</td>
<td>0.014</td>
</tr>
<tr>
<td>Hyperlipidaemia</td>
<td>1.94 (1.35–2.78)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Apoprotein A (per SD)</td>
<td>0.80 (0.67–0.95)</td>
<td>0.013</td>
</tr>
<tr>
<td>Lipoprotein (a) (per SD)</td>
<td>1.59 (1.35–1.87)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>hsCRP (per SD)</td>
<td>1.35 (1.15–1.58)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>CTRP1 (per SD)</td>
<td>1.73 (1.43–2.09)</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

OR (95% CI), odds ratio (95% confidence interval) for significant coronary artery disease; SD, standard deviation; hsCRP, high-sensitive C-reactive protein.

Model 1, adjusted for conventional cardiovascular risk factors;
Model 2, adjusted for the factors included in Model 1 with the addition of CTRP1.

Figure 1 Protein levels of C1q/TNF-related protein-1 in vascular samples and in peripheral blood mononuclear cells. (A) C1q/TNF-related protein-1 levels in coronary endarterectomy specimens (n = 32) and non-significantly atherosclerotic internal mammary artery tissue (n = 26) were assessed by Western blot and quantified by densitometry. (B) C1q/TNF-related protein-1 levels in aortic atherosclerotic plaque samples (n = 15) and non-significantly atherosclerotic aortic tissues (n = 10) were assessed by Western blot and quantified by densitometry. (C) C1q/TNF-related protein-1 in peripheral blood mononuclear cells in severely atherosclerotic patients (n = 58) and healthy controls (n = 25) were assessed by Western blot and quantified by densitometry.
Figure 2  C1q/TNF-related protein-1 induction of vascular cell adhesion protein-1, intercellular adhesion molecule-1, E-selectin, tumour necrosis factor-α, and monocyte chemoattractant protein-1 protein expression in human aortic endothelial cells, human umbilical vein endothelial cells, human native monocytes, and THP-1 monocytoid cells. Human aortic endothelial cells, human umbilical vein endothelial cells, human monocytes, and THP-1 monocytoid cells were stimulated with C1q/TNF-related protein-1 at increasing concentrations (0, 10, 100, and 1000 ng/mL) for 24 h. (A) Proteins levels of vascular cell adhesion protein-1, intercellular adhesion molecule-1, E-selectin, tumour necrosis factor-α, and monocyte chemoattractant protein-1 in endothelial cells were analysed by Western blot. (B) Protein levels of tumour necrosis factor-α and monocyte chemoattractant protein-1 in human monocytes and THP-1 cells were analysed by Western blot. (C–H) Quantifications of adhesion molecule protein levels in human aortic endothelial cells and human umbilical vein endothelial cells were done by band densitometry. (I–L) Quantifications of tumour necrosis factor-α and monocyte chemoattractant protein-1 levels in human monocytes and THP-1 cells were performed by band densitometry. Results are expressed as mean ± SD of six independent experiments. *P < 0.05; **P < 0.01 vs. control.
203580, but not inhibitors of ERK (PD98059) or of JNK (SP600125), virtually abolished CTRP1-induced p65 phosphorylation and the expression of VCAM-1, ICAM-1, and E-selectin in HAECs (see Supplementary material online, Figure S12), suggesting that CTRP1 is a pro-inflammatory adipokine acting by activation of the p38/NF-κB pathway.

**C1q/TNF-related protein-1 promotes leucocyte-endothelium interactions in vitro and in vivo**

We tested whether CTRP1 could induce leucocyte adhesion to the endothelium, which is a pathogenetic step in inflammation and atherogenesis. Results of these experiments, proving the hypothesis, are shown in the Supplementary material online, Figures S13–S15.

**Immunohistochemical detection of C1q/TNF-related protein-1 in atherosclerotic plaques**

To assess the relevance of our findings for the pathogenesis of atherosclerosis, we investigated CTRP1 expression in human atherosclerotic plaques. We obtained atherosclerotic femoral artery specimens from patients suffering from low-extremity artery occlusion and embolism, and also from patients undergoing low-extremity amputation because of traffic accident. We performed...
immunohistochemical staining on these tissues. Examination of atherosclerotic lesions revealed that CTRP1 was substantially expressed in the atherosclerotic intima, as well as in the cap and shoulder of advanced plaques (Figure 3), whereas its expression was much lower in non-atherosclerotic vessel counterparts (see Supplementary material online, Figure S16). Moreover, CTRP1 expression co-localized with CD68, suggesting that CTRP1 is involved mostly in macrophage and macrophage-derived foam cell-related processes in atherosclerotic plaques (Figure 3A–L). The expression of VCAM-1 was observed mostly...
in the endothelium of plaque microvessels, as marked by VE-cadherin, and in such areas CTRP1 was also co-expressed (Figure 3H and M–P).

Intraperitoneal injection of C1q/TNF-related protein-1 significantly promotes atherogenesis in apoE/−/− mice

To test the hypothesis of a pro-atherogenic effect of CTRP1 in vivo, we injected recombinant mouse CTRP1 protein (200 μg/kg, ~5 μg per mouse) (CTRP1 group, n = 12) or saline as control (saline group, n = 12) intraperitoneally every other day in apoE/−/− mice fed high-fat chow. Mice of both groups were then sacrificed at 24 weeks. During this experiment, one mouse in the CTRP1 group died of peritoneal infection. After 24 weeks, the atherosclerotic lesion area increased by 1.5-fold (p = 0.002) (see Supplementary material online, Figure S17).

Causal role of C1q/TNF-related protein-1 in atherogenesis and vascular inflammation: atherosclerosis and vascular inflammation are significantly attenuated in CTRP1/−/−/apoE/−/− mice compared with apoE/−/− control mice

To finally prove the causal role of CTRP1 in atherosclerosis and vascular inflammation, we constructed CTRP1/−/− mice and CTRP1/−/−/apoE/−/− double-knockout mice. ApoE/−/− (n = 12) and CTRP1/−/−/apoE/−/− double-knockout mice groups (n = 12) were fed high-fat chow for 24 weeks. After completion of this treatment period, we analysed the aortas of both mouse groups and found that the total plaque area was significantly lower in double-knockout mice than in apoE/−/− controls (Figure 4A and B). Levels of adhesion molecules (VCAM-1, ICAM-1, and E-selectin), TNF-α, phosphorylated-p38 MAPK, and phosphorylated-p65 were clearly lower in aortic tissues from CTRP1/−/− and CTRP1/−/−/apoE/−/− mice when compared with C57BL/6 and apoE/−/−, respectively (for all these comparisons, P < 0.05) (Figure 4C–I). These results were supported by findings from en face immunohistochemical staining, which revealed a robust VCAM-1 expression in the aortic endothelium of C57BL/6 mice, but very mild such expression in CTRP1/−/− mice after 24 weeks of high-fat diet; and by the co-localization of CTRP1 with the endothelium marked by VE-cadherin (Figure 4F). At immunohistochemistry, aortic plaques of double-knockout mice had significantly reduced CD45-, MOMA-2-, Ki67-positive areas, but increased α-SMA and collagen staining when compared with those of apoE/−/− mice (for all comparisons, P < 0.05), indicating attenuated atherogenesis by CTRP1 deletion (see Supplementary material online, Figures S18 and S19).

Double-knockout mice had higher body weight than apoE/−/− mice, but the difference did not achieve statistical significance. However, we found a significant difference in serum TNF-α levels between apoE/−/− and double-knockout mice (P = 0.016, see Supplementary material online, Table S3). We also cultured primary aortic endothelial cells and peritoneal macrophages from CTRP1/−/− and C57BL/6 mice, and treated these cells with TNF-α (50 ng/mL) to examine the CTRP1-mediated pro-inflammatory reaction. TNF-α-induced expression of adhesion molecules and of MCP-1 in endothelial cells, as well as of MCP-1 in macrophages were greatly attenuated by CTRP1 deletion when compared with similar cells from C57BL/6 (for all these comparisons, P < 0.05, Figure 4K–Q and Supplementary material online, Figure S20).

Discussion

The present study shows that (i) CTRP1 levels are significantly elevated in sera, coronary endarterectomy samples, atherosclerotic plaques and in PBMCs from patients with severe CAD when compared with controls and non-atherosclerotic samples; (ii) CTRP1 induces adhesion molecule expression and inflammatory cytokine production, and promotes leucocyte adhesion to endothelial cells in vitro and in vivo; (iii) the intraperitoneal injection of CTRP1 promotes atherogenesis in apoE/−/− mice; (iv) atherogenesis and vascular inflammation are significantly attenuated in CTRP1-deficient apoE/−/− mice. These results jointly suggest that CTRP1 is an adipokine contributing to atherogenesis and CAD.

C1q/TNF-related protein-1 is an adipokine secreted by the adipose tissue. However, in our study, no significant difference in BMI was present between controls and CAD patients. This result is consistent with another study in a similar population reported

**Figure 4** Atherosclerosis and vascular inflammation are attenuated in C1q/TNF-related protein-1/−/−/apoE/−/− mice. ApoE/−/− (n = 12) and C1q/TNF-related protein-1/−/−/apoE/−/− mice (n = 12) were fed a high-fat diet for 24 weeks; afterwards, atherosclerosis and atherogenic factors were analysed in the aortas of these mice. For experiments using mouse tissue or mouse-derived cells, data represent the average of ≥6 experiments per group. For animal experiments, (A) Representative photographs and software elaboration of oil red-O-stained mouse aortas of both groups: here red areas indicate positive staining for atherosclerotic lipids. (B) Quantification of atherosclerotic lesions (red areas) shown as percentage of the whole aortas. (C–I) Tumour necrosis factor-α (50 ng/mL) was used to stimulate peritoneal macrophages from C57BL/6 and CTRP1/−/− mice, with VE-cadherin as endothelial marker. (K) Tumour necrosis factor-α (50 ng/mL) was used to stimulate primary endothelial cells from C57BL/6 and CTRP1/−/−, after which the expression of tumour necrosis factor-α treatment.
by our hospital, whereby levels of another adipokine, adipocyte fatty acid binding protein, were found the difference between healthy controls and CAD despite no difference in BMI. Some, but not all, previous studies have supported a positive association of BMI and CAD. We rather believe, based on our findings, that increased serum CTRP1 levels in the patients of our current study are determined by the overall inflammatory status, including that of the adipose tissue, rather than the adipose tissue mass. Future studies should investigate whether major adipose tissues sources in some CAD patients are more capable of producing pro-inflammatory adipokines than in non-CAD patients. Meanwhile, the overall volume of the adipose tissue in our CAD patients does not account for differences observed compared with non-atherosclerotic controls.

In our animal studies, the intraperitoneal injection of CTRP1 (at 200 µg/kg every other day) caused an ~1.7-fold elevation of CTRP1 and a 1.8-fold elevation of serum TNF-α levels at 24 weeks, while knockout of CTRP1 significantly decreased serum TNF-α levels by 1.9-fold. Importantly, CTRP1 treatment significantly increased the percentage of atherosclerotic areas in the aorta of apoE−/− mice. Deletion of CTRP1 suppressed atherosogenesis compared with control mice. Our experiments also showed that CTRP1 treatment induces augmented expression of adhesion molecules and inflammatory factors in the aorta, and higher levels of macrophage infiltration in atherosclerotic plaques. All such features were reverted towards control levels in CTRP1−/+/apoE−/− double-knockout mice. Moreover, TNF-α-induced expression of adhesion molecules and MCP-1 in cultured endothelial cells and MCP-1 in peritoneal macrophages were all attenuated by CTRP1 deletion, suggesting a positive autocrine feedback-loop by TNF-α/CTRP1 stimulation, leading to an even stronger inflammatory activation in such conditions. These results provide substantial evidence to support data of patient samples, indicating that CTRP1 exerts crucial pro-inflammatory and pro-atherogenic effects in vivo.

Our study showed that CTRP1 up-regulates the expression of adhesion molecules through the p38 MAPK/NF-κB pathway, and that the p38 MAPK inhibitor SB203580 remarkably inhibits CTRP1-induced adhesion molecule expression and monocytoid cell-endothelial cell interaction. Such results are consistent with previous studies, indicating that activation of the p38 MAPK/NF-κB pathway enhances the expression of adhesion molecules in endothelial cells. Previous studies have also reported that the p38 MAPK/arginase pathway mediates angiotensin II-induced endothelial dysfunction, and that arginase inhibition improves endothelial function in patients with CAD and diabetes mellitus. Moreover, in our study, compared with controls, phosphorylated-p38 MAPK was significantly elevated in atherosclerotic aortic tissue from apoE−/− mice treated with CTRP1 injections, but was lower in aortic tissues from CTRP1−/− or double-knockout mice. Thus, these findings jointly suggest that the p38 MAPK pathway is crucial to mediate pro-inflammatory and pro-atherogenic effects of CTRP1.

We acknowledge that additional studies are needed to clarify the physiology and pathophysiology of CTRP1. For instance, it is still unclear why this protein, featuring pro-inflammatory properties, also has antithrombotic effects, which CTRP1-expressing cell type is responsible for promoting atherosclerosis; and whether treatments targeting CTRP1 will induce plaque regression or prevent further progression of established atherosclerosis. All this deserves further investigation. We also recognize that a limitation in our patient in vivo observational study is its cross-sectional nature, thereby allowing to detect associations of serum concentrations of this adipokine with CAD, but not to formulate risk predictions.

Conclusions

The present study demonstrates that increased levels of CTRP1 mark the extent and severity of atherosclerosis in human subjects, and that this adipokine has a causal role for atherosclerosis in vitro and in vivo mice.

Supplementary material

Supplementary Material is available at European Heart Journal online.

Authors’ contributions


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References


