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Effects of the new adiponectin paralogous protein CTRP-3 and of LPS on cytokine release from monocytes of patients with type 2 diabetes mellitus

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ABSTRACT

Aims/Hypothesis: It was the aim to investigate the hypothesis that the new C1q/TNF-family member CTRP-3 (C1q/TNF-related protein-3) acts anti-inflammatory in human monocytes from healthy controls and patients with type 2 diabetes mellitus (T2D). *Methods:* Monocytes were isolated from 20 healthy controls and 30 patients with T2D. IL-6 and TNF concentrations were measured by ELISA. CTRP-3 was expressed in insect cells and used for stimulation experiments. *Results:* Basal IL-6 and TNF were not different in control and in T2D monocytes. LPS-stimulation (1 μg/ml) significantly ($p < 0.001$) increased IL-6 and TNF in the supernatants of control and in T2D monocytes to a similar extent. CTRP-3 (1 μg/ml) significantly ($p = 0.03$) inhibited LPS-induced IL-6 in control monocytes but not in T2D monocytes. TNF upon co-stimulation with LPS and CTRP-3 was significantly ($p = 0.012$) lower in control than in T2D monocytes. LPS-induced TNF concentration was significantly and positively correlated with serum total cholesterol and LDL cholesterol in T2D patients. *Conclusions:* CTRP-3 inhibits LPS-induced IL-6 and TNF release. This anti-inflammatory effect is lost in T2D. Serum cholesterol concentration affects the pro-inflammatory potential of LPS to induce TNF release from T2D monocytes in the presence or absence of CTRP-3. CTRP-3 might partly account for the pro-inflammatory state in T2D.

1. Introduction

There are several lines of evidence for the involvement of adipose tissue in innate and acquired immune responses [1,2]. Adipocytes are potent producers of chemokines and pro-inflammatory cytokines, such as interleukin-6 (IL-6) and tumor necrosis factor (TNF). Furthermore, adipocytes secrete high amounts of adipokines such as leptin, adiponectin, and resistin that regulate monocyte/macrophage function. Finally, adipocytes secrete novel molecules that are associated with the innate immune system. These novel molecules are classified using the term C1qTNF-related protein (CTRP) superfamily.

Proteins with a C-terminal complement factor C1q globular (gC1q) domain that has a three-dimensional structure similar to that of TNF, C1q and adiponectin are designated as CTRP's [3]. These proteins share a similar and modular organization to adiponectin and comprise four distinct domains, i.e., a N-terminal signal peptide, a short variable region, an N-terminal collagenous domain with various length of Gly-X-Y repeats, and a C-terminal C1q globular domain. These family members developed by divergence from a common precursor molecule of the innate immune system during evolution. There is an expanding family of proteins with a gC1q domain. When the NCBI (National Center for Biotechnology Information) database is searched with the adiponectin cDNA sequence, the cDNA of 7 family members can be found, CTRP-1, CTRP-2, CTRP-3, CTRP-4, CTRP-5, CTRP-6, and CTRP-7 [2,4]. Expression, regulation and function of these new family members are largely unknown.

Importantly, some of the secreted CTRP's are highly expressed and secreted by adipose tissue such as CTRP-3 [5–9]. This observation directly links the adipose tissue to inflammation, apoptosis, autoimmunity, host defense, hibernation, organogenesis, cell differentiation, metabolism, and insulin signaling. In the case of CTRP-3, data are still rare, but there is increasing evidence for immuno-regulatory effects of this molecule on adipocytes [8] or monocytes [10] and proliferative effects on soft tissue cells [11,12] and endothelial cells [13].

Adipocyte-derived mediators are known to affect the pro-inflammatory response of monocytes and the pro-inflammatory response of immune cells is affected in type 2 diabetes mellitus (T2D). Since it is our hypothesis that CTRP-3 acts anti-inflammatory on human monocytes, it was the aim of the present study.

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- To investigate the effects of high dose LPS-stimulation and CTRP-3-stimulation alone or a combination of both on the release of IL-6 and TNF in primary human monocytes isolated from healthy controls and patients suffering fromT2D.

Since serum lipoproteins are known to control LPS-induced monocyte activation and cytokine release [14], we further aimed

- To investigate whether basal or LPS-induced monocytic cytokine release depends on the respective serum lipoprotein levels in T2D patients.

2. Patients, material and methods

2.1. Study cohort

10 female and 10 male healthy medical probands (students at the University Hospital of Regensburg, Germany) served as controls ($n = 20$). Thirty patients (11 females and 19 males) suffering from T2D were included in the study. All individuals participating in the study gave informed consent, and the study was approved by the local ethical committee. The characteristics of the entire study population are summarized in Table 1. Patients suffering from acute/chronic infections and chronic inflammatory diseases were excluded. Patients with T2D were significantly older and had a higher BMI when compared to our control population.

2.2. Isolation and purification of human primary monocytes

Whole blood was drawn from controls and patients after an overnight fast using the Vacutainer CPT System (Becton–Dickinson, Franklin Lakes, NJ, USA). Primary human monocytes were isolated and cultured as described previously [15] using magnetic separation with CD14 beads (Miltenyi Biotec., Bergisch-Gladbach, Germany). Briefly, monocytes were cultured in RPMI 1640 medium (Biochrom AG, Southborough, MA, USA) supplemented with 10% autologous serum for 18 h. Since the average quantity of isolated monocytes per individual was limited to the range of 12–13×10^6 monocytes, a maximum of four sets of experiments was possible for each individual. Thus, for each stimulation experiment, 3×10^6 monocytes were used. Prior to the stimulation experiments, the medium was replaced. Unstimulated monocytes served as a control. Monocytes were stimulated for 12 h with LPS (1 μg/ml) alone, CTRP-3 (1 μg/ml) alone, and with a combination of LPS (1 μg/ml) and CTRP-3 (1 μg/ml). Since obesity and insulin resistance can be regarded as a chronic and low grade state of inflammation and T2D patients are potentially used to some kind of chronic immune stimulation, we decided to use a high dose of LPS (1 μg/ml). When a combination of LPS and CTRP-3 was used, CTRP-3 was added to the cells 30 min. prior to LPS in order to allow CTRP-3 to exert potential anti-inflammatory effects. LPS (*E. coli*, serotype 055:B5) was purchased from Sigma–Aldrich (Deisenhofen, Germany).

Table 1
Characteristics of the study population.

	Controls ($n = 20$)	Type 2 diabetes mellitus ($n = 30$)
Females (n %)	10 (50%)	11 (36.7%) n.s.
Males (n %)	10 (50%)	19 (63.3%) n.s.
BMI (kg/m^2)	21.9 + 0.3	31.0 + 12.7[a] ($p < 0.01$)
Age (years)	25.1 + 1.5	62 + 11.8[b] ($p < 0.01$)
Lipoprotein-metabolism		
Cholesterol (mg/dl) [range]	–	156 + 48 [40–244]
Triglycerides (mg/dl) [range]	–	129 + 59 [56–279]
HDL (mg/dl) [range]	–	40 + 13 [17–88]
LDL (mg/dl) [range]	–	88 + 37 [9–146] [8 (27%) patients were on statins]
Carbohydrate-metabolism		
HbA_{1c} (%) [range]	–	7.5 + 2.8 [4.6–18.0]
Fasting glucose (mg/dl) [range]	–	157 + 92 [81–500]
Inflammation		
CRP (mg/l)	–	29 + 46
WBC (/nl)	–	8.0 + 3.4
Anti-diabetic treatment		
Diet/exercise n (%)	–	3 (10%)
Oral treatment n (%)	–	9 (30%)
Insulin n (%)	–	14 (46.7%)
Insulin + oral treatment n (%)	–	4 (13.3%)

[a] BMI in controls vs. BMI in patients with type 2 diabetes mellitus ($p < 0.01$).
[b] Age in controls vs. age in patients with type 2 diabetes mellitus ($p < 0.01$). Mean values + SD and ranges are shown.

2.3. Recombinant CTPR-3 expression

Since CTRP-3 represents a novel adipokine, the recombinant protein is currently not available on a commercial basis. Therefore, recombinant CTRP-3 protein expression was performed in H5 insect cells (Invitrogen, Karlsruhe, Germany) using the BacPAK™ Baculovirus Expression System (BD Biosciences, Palo Alto, CA, USA) as published earlier by our group in detail [10]. Supernatants were collected 3 days after infection for purification of CTRP-3 using the BD Talon TM Purification kit (BD Biosciences). Integrity and purity of the protein were analyzed by immunoblot and silver staining of SDS–PAGE. In contrast to *E. coli-based* expression systems, the recombinant expression in insect cells usually maintains glycosylation and phosphorylation. Based on this, we could demonstrate earlier that our expression system generates trimeric CTRP-3 that was used for stimulation experiments [10,16].

2.4. Measurement of supernatant cytokine and chemokine concentration of human monocytes

The concentration of IL-6 and TNF was measured by ELISA. All ELISA-based detection systems were purchased from R&D systems, Wiesbaden, Germany (DuoSet ELISA development systems). For data normalization, total protein concentration was measured. For the detection of potential cytotoxic effects, LDH concentration was measured in the supernatants (cytotoxicity detection kit, Roche, Mannheim, Germany). Each sample was measured in duplicate by ELISA. Values were normalized to total protein content of each well and were expressed as means + standard error of the mean.

2.5. Western blot analysis

SDS–polyacrylamide gel electrophoresis was performed following standard procedures. Proteins were transferred to a PVDF Fluotrans Transfer Membrane (Pall Corp., Portsmouth, England). Transfer was confirmed by Ponceau Red staining of the membrane and Coomassie Blue staining of the gel after the electroblot. Detection of the immune complexes was carried out with the enhanced chemiluminescence Western blot detection system (Amersham ECL™ Western blotting system, GE Healthcare, Freiburg, Germany). The anti-*PI3K p85* antibody was used as a loading control (Upstate Biotechnology, Lake Placid, NY, USA) with a 1:1000 dilution. The expression of Erk (anti-MAP-kinase-1/2) and phospho-Erk (anti-phospho-p44/p42 MAP-kinase [Thr202/Tyr204]) was investigated using monocytes from 4 healthy controls. The *erk* and *phospho-erk* antibodies were used with a 1:1000 dilution. The secondary peroxidase-coupled anti-rabbit antibody (donkey) was used with