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Increased levels of inflammatory mediators and proinflammatory monocytes in patients with type I diabetes mellitus and nephropathy

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ABSTRACT

Aims: To investigate and describe the relationship between diabetic nephropathy and systemic inflammation in patients with type 1 diabetes mellitus (T1DM).
Methods: Patients with T1DM, with or without reduced renal function due to diabetic nephropathy, were included. Differences in inflammatory mediators, adhesion molecules, markers of endothelial dysfunction and subsets of monocytes were studied in patients with mean disease duration of 31 years.
Results: Patients with T1DM with and without renal failure were compared. Patients with nephropathy had increased plasma levels of proinflammatory monocytes, as well as circulatory PAI-1, syndecan-1, VEGF, IL-1β, IL-1Ra and CCL4. Peripheral blood mononuclear cells from patients with nephropathy numerically increased soluble ICAM and PAI-1 in co-culture with primary endothelial cells compared to cells from patients without nephropathy.
Conclusions: T1DM patients with kidney failure have higher levels of proinflammatory monocytes and circulatory inflammatory mediators compared to patients with T1DM alone. The results highlight the importance of inflammation and endothelial dysfunction in diabetic nephropathy with reduced GFR.

1. Introduction

The complications of type 1 diabetes mellitus (T1DM) represent a significant burden to human health, and the prevalence of T1DM is increasing worldwide (Daneman, 2006). Long-term complications can be divided into two categories; macro- and microvascular. General risk factors for diabetic complications are poor glycemic control (The Diabetes Control and Complications Trial Research Group, 1993; UK Prospective Diabetes Study (UKPDS) Group, 1998 Nathan et al., 2005), smoking, obesity, hypertension, hyperlipidemia, genetic predisposition and early onset of T1DM (Daneman, 2006).

Cardiovascular disease (CVD) represents the macrovascular complications in diabetes. The relative risk of CVD in T1DM is reported to be 10-fold higher compared to the non-diabetic population (Daneman, 2006). Diabetic nephropathy is a microvascular complication of diabetes, and the leading cause of end stage renal failure (ESRD) in the western world (Gilbertson et al., 2005) and Asia (Tang, 2010). Nephropathy is clinically demonstrated by hypertension, albuminuria and increased decline of the glomerular filtration rate (GFR). Chronic kidney disease (CKD) is a serious condition where inflammation and endothelial dysfunction are important components (Recio-Mayoral, Banerjee, Streather, & Kaski, 2011), and a risk factor for CVD and all-cause mortality (Heine et al., 2012; Weiner et al., 2004). Hypertension and poor glycemic control precede the development of nephropathy which is also accompanied by an unfavorable lipid profile (Hirano, 2014). Increased levels of inflammatory circulating mediators have been shown to predict the onset and progression of nephropathy and other long-term complications (Forbes & Cooper, 2013).

Monocytes are a heterogenous group of cells, and can be subdivided into three different subtypes based on their expression of CD16 and CD14 on their surface. The subsets possess different phenotypes and functions (Wong et al., 2012). The major fraction of circulating monocytes are classical CD14-positive and CD16-negative (CD14++CD16−) monocytes (Scherberich & Nockher, 1999). CD16 enriched monocytes (CD14++CD16+, referred to as intermediate monocytes and CD14 + CD16++ referred to as non-classical monocytes) are all together termed proinflammatory monocytes (Passlick, Flieger, & Ziegler-Heitbrock, 1989; Wong et al., 2012; Ziegler-Heitbrock et al., 2010). Expanded levels of monocytes enriched in CD16 have been found in patients with ESRD on dialysis (Nockher & Scherberich, 1998; Ulrich, Heine, Seibert, Fliser, & Girndt, 2010) and with coronary artery disease (Tallone et al., 2011). CD16-enriched monocytes from patients with CKD of various causes have increased ability to adhere to endothelial cells. Monocyte activation is referred to as a cellular hallmark of chronic inflammation, and contributes to increased risk of CVD in patients

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with CKD (Heine et al., 2012). Especially, CD16-positive monocytes have shown increased ability to secrete proinflammatory mediators (Belge et al., 2002; Frankenberger, Sternsdorf, Pechumer, Pforte, & Ziegler-Heitbrock, 1996; Ziegler-Heitbrock, 2007) and to adhere to endothelial cells (Ramirez et al., 2011), relevant for patients with ESRD (Nockher & Scherberich, 1998; Ulrich et al., 2010).

Dysfunction of the vascular endothelium is an important factor in the pathogenesis of diabetic complications (Brownlee, 2001; De Vriese, Verbeuren, Van de Voorde, Lameire, & Vanhoutte, 2000). Low grade inflammation in diabetes is supported by endothelial cells through their synthesis of inflammatory molecules such as cytokines, chemokines, adhesion molecules, proteoglycans and other secretory products (Sumpio, Riley, & Dardik, 2002).

The aim of this study was to investigate whether there are differences between patients with T1DM with or without diabetic nephropathy with renal failure with respect to inflammatory mediators and proinflammatory monocytes. The results may reflect the impact of nephropathy on systemic inflammation in patients with T1DM and can also reflect a difference in the regulation of inflammatory mediators between these patient groups before the development of nephropathy.

2. Subjects, Materials and Methods

2.1. Subjects

Twenty-eight patients with T1DM were included in this study. Thirteen of them had kidney failure (the DN group) and 15 had normal renal function (The D group). Kidney failure was defined as estimated glomerular filtration rate (eGFR) below 40 ml/min/1.73m^2 and normal renal function was defined as eGFR > 60 ml/min/1.73m^2. Mean albumin/creatinine ratio was 249.9 mg/mmol in the DN group (data missing on four patients with eGFR levels lower than 33 ml/min/1.73m^2) and 2.2 mg/mmol in the D-group (unknown value for three of these patients). Results after inclusion show that the highest eGFR measured in the DN group was 34 ml/min/1.73m^2, the lowest 12.5 ml/min/1.73m^2.

Inclusion took place at Akershus University Hospital, Norsk Diabetikersenter (an outpatient clinic for patients with T1DM) and Oslo University Hospital Ullevaal. The patients donated between 40 and 50 ml of venous blood. Age, body mass index, sex, regular medication and diabetes age were recorded, as well as their history of CVD. Blood pressure was measured before blood sampling.

All of the DN patients were on antihypertensive treatment. No data on the type of antihypertensive medication were available. In the D group, only six patients received antihypertensive drugs, some of them angiotensin converting enzyme inhibitors or angiotensin II receptor blockers. Five of the patients in the DN group were treated with acetylsalisylic acid (ASA), and one of them with clopidogrel. In the D group, three patients used ASA daily. Nine out of thirteen DN patients received cholesterol lowering medication, while only four out of fifteen in the D group received this type of drugs. Self-reported cardiovascular disease was present in two of the patients in the DN group and one of the patients in the D group. Three patients were excluded from the study. Exclusion criteria were ongoing renal replacement therapy, eGFR between 40 and 60 ml/min/1.73m^2, hemoglobin levels below 10 mg/dl, signs of ongoing infections, CRP-levels above 15 mg/ml, or ongoing treatment with immunosuppressive medication. HbA1c, white blood cell counts, cholesterol levels, CRP, creatinine (and thereby estimation of eGFR) were measured in the clinics by their routine laboratories.

2.2. Ethics Statement

This patient study was approved by the regional ethics committee for Southern Norway, and followed the guidelines of the Helsinki declaration. All patients, both T1DM patients and donors of umbilical cords, signed informed consent.

2.3. Inflammatory Mediator Assays

CRP and white blood cell counts in patient blood were determined by standard clinical laboratory methods. Plasma was obtained from heparinized patient blood stored on ice for a maximum of 60 min. Blood cells were pelleted by centrifugation (3000 rpm, 10 min), and plasma were collected and frozen at −20 °C.

Plasma was analyzed with a selected 10-plex human cytokine/chemokine panel (a multiplex antibody bead kit) targeting TNFα, MMP-9, IL-1β, IL-6, IL-8, IL-10, CCL2, CCL3, CCL4 and IL-1Ra in addition to human Serpin E1/PAI-1 and VEGF Quantikine ELISA Kit, Human IL-1F2 QuantiGlo ELISA Kit, Human ICAM-1/CD54 Duoset-1 kit (all from R&D systems), and Syndecan-1 ELISA kit (Diaclone). The same ELISA kits were used for measurements performed on cell media/supernatants. All ELISAs were performed according to the manufacturer's instruction, and all standards were within the limits of detection.

2.4. Peripheral Blood Mononuclear Cell (PBMC) Isolation

Peripheral vein blood was obtained from patient blood using heparin tubes kept at 37 °C for a maximum of 60 min. Peripheral blood mononuclear cells were isolated by density gradient centrifugation, using Polymorphprep (Axis-Shield Poc AS) centrifugation according to the manufacturer's instructions. The PBMCs were suspended in RPMI (Sigma) with antibiotics (penicillin/streptomycin), 10% fetal calf serum and L-glutamine prior to RNA isolation, or adherence to endothelial cells. PBMCs were also resuspended in 0.5 ml 50% FCS in RPMI and subsequently 0.5 ml of 20% DMSO in RPMI before being frozen at −80 °C for later flow cytometry analysis.

2.5. Flow Cytometry and Cell Sorting

CD14+ and CD16+ cells from PBMC patient samples were quantified. All monocytes are CD14-positive (CD14+), but all CD14 + CD16−, CD14 + CD16+ and CD14−CD16+ cells were counted. PBMC were blocked with FcR blocking reagent from Miltenyi biotec (Bergisch Gladbach, Germany) before staining with anti-CD16 (3G8-Alexa647) and anti-CD14 (M5E2-FITC) from BD Pharmingen (Franklin Lakes, NJ). Isotype antibodies, mouse IgG1 (MOPC-21-Alexa647) and mouse IgG2a (G155–178-FITC) from BD Pharmingen were used to exclude unspecific binding. Following this, cells were stained with Fixable Viability Dye eFluor 506 from eBioscience (San Diego, CA) to gate out dead cells. Cells were then fixed in 2% paraformaldehyde for 10 min. Samples were analyzed using the BD Canto (BD Biosciences) and data analysis was performed with FlowJo 7.6.5. software.

2.6. The Adherence Model

2.6.1. Endothelial Cell Culture

Primary human umbilical vein endothelial cells (HUVEC) were isolated from umbilical cords as described (Jaffe, Nachman, Becker, & Minick, 1973). Cells were cultured at 37 °C and 5.0% CO_2 in MCDB 131 medium (Sigma) containing 5 mM glucose and supplemented with 7% heat-inactivated fetal calf serum (FCS, Sigma), basic fibroblast growth factor (bFGF, 1 ng/ml, R&D), hydrocortisone (1 μg/ml, Sigma), epidermal growth factor (EGF, 10 ng/ml, R&D), gentamicine (50 μg/ml, GIBCO Invitrogen) and fungizone (250 ng/ml, GIBCO Invitrogen). The medium was replaced three times a week and cells were used for experiments within three passages. The purity of the endothelial cell cultures was verified by microscopic observations of each culture as well as regular staining for the endothelial cell marker von Willebrand factor (vWF).

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