Available online at www.sciencedirect.com
ScienceDirect
Biochemical and Biophysical Research Communications 362 (2007) 238–244
BBRC
www.elsevier.com/locate/ybbrc

Impact of diabetic serum on endothelial cells: An in-vitro-analysis of endothelial dysfunction in diabetes mellitus type 2

Daniela Münzel [1], Karla Lehle [1,*], Frank Haubner, Christof Schmid, Dietrich E. Birnbaum, Jürgen G. Preuner

Department of Cardiothoracic Surgery, University Hospital Regensburg, D-93042 Regensburg, Germany

Received 5 June 2007
Available online 18 June 2007

Abstract

Diabetic endothelial dysfunction was characterized by altered levels of adhesion molecules and cytokines. Aim of our study was to evaluate the effects of diabetic serum on cell-growth and proinflammatory markers in human saphenous vein endothelial cells (HSVEC) from diabetic and non-diabetic patients. Diabetic serum showed (1) complementary proliferative activity for non-diabetic and diabetic HSVEC, (2) unchanged surface expression of adhesion molecules, and (3) elevated levels of sICAM-1 in HSVEC of all donors. The concentration of sVCAM-1 was increased only in diabetic cells. The proinflammatory state of diabetic HSVEC characterized by increased levels of cytokines was compensated. We concluded that even under normoglycemic conditions the serum itself contains critical factors leading to abnormal regulation of inflammation in diabetics. We introduced an in vitro model of diabetes representing the endothelial situation at the beginning of diabetes (non-diabetic cells/diabetic serum) as well as the diabetic chronic state (diabetic cells/diabetic serum).

Keywords: Endothelial cell; Diabetes mellitus; Diabetic serum; Cell-growth; Adhesion molecules; Cytokines

Cardiovascular disease is a major cause of morbidity and mortality in type 2 diabetes mellitus, and a cause of death in approximately three of four diabetic patients [1]. Diabetes is associated with a two- to fourfold increase in the risk of developing coronary artery disease (CAD). The risk of a myocardial infarction in diabetic patients without evident CAD is comparable to the risk of non-diabetic patients with a previous myocardial infarction [2]. A wide variety of studies suggest that the endothelium is a major target organ of cardiovascular risk factors such as hypercholesterolemia, hypertension, infection, ageing, smoking and diabetes per se [3]. The vascular endothelium plays a critical role in modulating both the inflammatory response and vasomotor abnormalities seen in atherosclerosis and diabetes. Damage to the endothelium leads to endothelial dysfunction, characterized by decreased endothelium-dependent vasorelaxation, increased leucocyte-endothelial cell adhesion, vascular permeability and prothrombotic properties [4,5]. In this proinflammatory state, endothelial cells show an upregulation of adhesion molecules and increased release of chemokines (e.g., MCP-1) [5]. Elevated concentrations of these molecules in the peripheral blood of diabetic patients demonstrate the extent of the dysfunction [6–9]. There are several techniques to evaluate endothelial dysfunction in vivo, such as coronary artery reactivity to acetylcholine or brachial artery ultrasonography [10]. The dysfunction of diabetic endothelial cells was also demonstrated in our recently published in vitro model [11]. In search of the main causes of this endothelial dysfunction the effects of high glucose

Abbreviations: EC, endothelial cells; IL-6, interleukin-6; IL-8, interleukin-8; MCP-1, monocyte chemoattractant protein-1; sVCAM, soluble vascular cellular adhesion molecule-1; sICAM, soluble intercellular adhesion molecule-1.

* Corresponding author. Fax: +49 (0)941 9449902.
E-mail address: Karla.Lehle@klinik.uni-regensburg.de (K. Lehle).

[1] These authors contributed equally to this work.

0006-291X/$ - see front matter © 2007 Elsevier Inc. All rights reserved.
doi:10.1016/j.bbrc.2007.06.045

concentrations [12–14], of advanced glycation end products (AGES) [15–19], of soluble CD40-ligand [20], and of additional, so far unknown factors in the serum of diabetic patients were discussed.

The aim of the present study was to investigate the impact of diabetic serum on the proinflammatory properties of HSVEC from non-diabetic and diabetic cell donors in our in vitro system.

Materials and methods

Cell culture. Endothelial cells from saphenous veins (HSVEC) were prepared from type 2 diabetic ($n = 11$) and non-diabetic ($n = 8$) patients undergoing coronary artery bypass surgery. Patients with diabetes mellitus in their medical history of more than 10 years and fasting glucose levels $\geqslant 7.0$ mM were consulted as diabetic cell donors. Informed consent was obtained from all patients. The protocol for isolation of endothelial cells and the use of human serum from diabetic patients were approved by the Ethical Committee of the University of Regensburg (No. 99/133 and 04/189).

HSVEC were isolated and cultured according to Jaffe et al. [21], as previously described [11]. Briefly, primary endothelial cells were detached from human saphenous veins by collagenase treatment, resuspended in culture medium containing Endothelial Cell Basal Medium (PromoCell® Heidelberg, supplemented with 2% fetal bovine serum, 0.4% ECGS/h, 0.1 ng/ml hEGF, 1 ng/ml hbFGF, 1 μg/ml hydrocortisone, 50 μg/ml gentamycin, 50 ng/ml amphotericin B) and 30% pooled human serum and cultured on gelatin-coated tissue culture flasks. After reaching confluence (8–14 days), the cultures were subcultured (passage 1) and finally the cells were cryopreserved in 10% dimethylsulfoxide until usage. Recultured HSVEC were characterized by their cobblestone structure monitored by phase contrast microscopy (Leica DMRBE, Bensheim, Germany), and by flow cytometric analysis using an anti-human CD31 antibody (Ancell, Bayport, MN, USA) staining. To consider the interindividual variability of primary cells, we carried out the experimental protocol with cells from different cell donors (Table 1).

For the experiments, HSVEC (passage 3) were seeded in 96-well-microtiter-plates (1200 cells/well) with 150 μl culture medium. After 3 days under standard culture conditions (=exponential growth phase), the cells were exposed to the following experimental conditions for an additional 3 days: (1) culture medium containing 30% human serum from diabetic patients (DM), (2) culture medium with 30% serum from healthy controls (HS). To exclude the concomitant effects of blood glucose and drugs (e.g., diabetic or cardiac medication) in the serum of diabetic subjects on endothelial cells, pooled diabetic serum was dialyzed against phosphate-buffered saline (3×5 l PBS, 4 °C, over night, pore size of dialysis hose 3.5 kDa).

Cell growth. Proliferating diabetic and non-diabetic HSVEC were incubated with DM (1) and HS (2), respectively. Cells counts were analyzed before and 24, 48, and 72 h after incubation. Therefore, cells were detached by collagenase digestion, and cell number was determined by CASY® Cell Counter and Analyser System, Model TTC (Schärfe Systems GmbH, Reutlingen, Germany).

Surface expression of adhesion molecules. Cellular surface expression of ICAM-1, VCAM-1, and E-selectin was quantified using cellular ELISA [11]. Briefly, fixed cells were blocked with 1% BSA for 1 h and incubated with mouse monoclonal antibodies [anti-human ICAM-1 and VCAM-1 antibody (DAKO, Glostrup, Denmark), anti-human E-selectin antibody (Ancell)]. Biotinylated anti-mouse antibody (Vector Laboratories Ltd., Peterborough, UK) was used for secondary detection. Alkaline phosphatase streptavidine (Vector) was added as conjugate, and finally para-nitrophenylphosphate (Sigma, Saint Louis, Missouri, USA) was applied as substrate. The extinction was measured at 405 nm using a microplate-reader (Molecular Devices, Union City, CA, USA).

Detection of soluble adhesion molecules and cytokines. The concentration of soluble adhesion molecules (ICAM-1, VCAM-1, and E-selectin) and interleukin-6 and -8 (IL-6, IL-8) and monocyte chemoattractant protein-1 (MCP-1) was detected in the supernatants of treated HSVEC (48 h) using commercially available ELISA-kits [Biosource (Nivelles, Belgium), R&D Systems (Wiesbaden, Germany), PromoCell® (Heidelberg, Germany), Beckman-Coulter (Krefeld, Germany)]. Previously, the content of cytokines and soluble adhesion molecules was detected in both diabetic (DM) and control (HS) serum and was set as a blank value, in order to quantify the cell-specific production of these cytokines and adhesion molecules.

Statistical analysis. All experimental parameters were prepared in quadruplicate. Data are presented as median including 25% and 75% percentile. Changes were analyzed using the Wilcoxon Signed-rank Test and the Mann–Whitney Rank Sum Test. A p-value $\leqslant 0.05$ was considered to be statistically significant.

Table 1
Patient characteristics of diabetic and non-diabetic cell donors

	Non-diabetic cell donors	Diabetic cell donors	p-value
n	8	11	—
Sex (M/F)	7/1	9/2	N.S.
Age (years)	64 ± 8	70 ± 7	N.S.
BMI (kg/m^2)	28 ± 2	28 ± 4	N.S.
Blood pressure			
Systolic (mm HG)	145 ± 25	152 ± 24	N.S.
Diastolic (mm HG)	80 ± 16	76 ± 13	N.S.
Total cholesterol (mg/L)	1832 ± 372	1846 ± 576	N.S.
Triglycerides (mg/L)	1194 ± 194	1434 ± 576	N.S.
LDL cholesterol (mg/L)	1014 ± 349	856 ± 208	N.S.
HDL cholesterol (mg/L)	514 ± 70	461 ± 123	N.S.

Results

Patients' characteristics of cell donors and donors of diabetic serum

As shown in Table 1, the demographic data and baseline clinical characteristics of non-diabetic and diabetic patients were comparable. Exempt from diabetes mellitus type 2, there were no differences concerning haemodynamics and their cardiovascular risk factors. The HbA1c values varied from 5.3 to 9.1 in the diabetic patients.

Diabetic serum was collected and pooled from 47 diabetic patients. Here, the mean age of subjects was 65.8 years (SD ± 9.4 years). Mean HbA1c value was 7.8 (SD ± 1.54). Frequent secondary disorders were CAD (55.3%), arterial hypertension (74.5%), neuropathy (17.0%), nephropathy (23.4%), retinopathy (12.8%), hyperlipidemia (34.0%), hypercholesterolemia (8.5%) and hyperuricemia (8.5%). Levels of cytokines and soluble adhesion molecules were quantified in pooled DM- and HS-serum as shown in Table 2.

Diabetic serum impaired endothelial cell growth kinetics

To test the impact of diabetic serum on cell growth, proliferating HSVEC from diabetic and non-diabetic cell donors were cultivated in DM-serum and HS (as a control) for 24 and 72 h. The ratio of cell counts after treatment relative to the cell counts at the beginning of serum incubation defines the cell multiplication factor (CMF). Seventy-two hours cultivation of non-diabetic HSVEC