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# Development of a disease-specific model to evaluate endothelial dysfunction in patients with diabetes mellitus

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**Abstract**

Diabetic patients have an increased cardiovascular risk. We propose to characterize the endothelial dysfunction in a disease-specific *in vitro* model. Human saphenous vein endothelial cells (HSVEC) were isolated from coronary artery bypass patients without and with non-insulin-dependent diabetes mellitus. Growth kinetics and proinflammatory responses (expression of adhesion molecules, cytokines) were documented under non-stimulating conditions. Diabetic HSVEC showed delayed growth kinetics with reduced cell densities of about 40%. During exponential growth of diabetic EC, the surface expression of adhesion molecules was increased 10-fold ($p \leqslant 0.05$). However, in a monolayer the expression adapted to low levels of non-diabetic EC. In addition, diabetic EC produced significantly more soluble E-selectin, VCAM-1, IL-6 and MCP-1. Our results suggest a link between the pathologically proinflammatory basic state of diabetic EC and the endothelial dysfunction in diabetic disease. Therefore, this *in vitro* model could be used for investigating early dysfunction and environmental effects on pathological endothelium.


*Keywords:* Endothelial dysfunction; *In vitro* model; Diabetes mellitus; Endothelial cells

Insulin-dependent (IDDM) and non-insulin-dependent diabetes mellitus (NIDDM) are associated with an increased risk for atherosclerotic cardiovascular disease [1]. Endothelial dysfunction, defined as an imbalance between relaxing and contracting factors, procoagulant and anticoagulant factors, and inflammatory and anti-inflammatory factors [2,3], are a condition sine qua non to vascular complications of diabetes mellitus [4]. The precise mechanisms regulating the initiation and progression of vascular lesions in diabetic patients remain unclear.

Inflammation, through overexpression of adhesion molecules and cytokines and leukocyte adhesion to endothelial cells, is thought to participate in the pathogenesis of atherosclerosis and also in the development of non-insulin-dependent diabetes mellitus [5]. It has been reported that exposure of vascular endothelium to elevated glucose concentration induces expression of adhesion molecules *in vitro* [6–8]. In addition, serum concentrations of soluble adhesion molecules (sE-selectin, sVCAM-1, sICAM-1) are elevated in patients with NIDDM [9–13].

In this study we presented for the first time a disease-related *in vitro* model to verify the endothelial dysfunction in the diabetic vascular disease.

## Materials and methods

*Cell culture.* EC from saphenous veins (HSVEC) were prepared from diabetic (NIDDM, $n = 8$) and non-diabetic ($n = 8$) patients undergoing coronary artery bypass surgery. Subjects who self-reported having diabetes mellitus type 2 up to ten years (3/8 with oral antidiabetics, 3/8 insulin, 2/8 dietetic adjusted) or who had fasting glucose levels $\geqslant 7.0$ mM were designated as having diabetes mellitus. Informed consent was obtained from each patient, and the protocol for isolation of endothelial cells was approved by the Institutional Review Board of the University of

*Abbreviations:* EC, endothelial cells; NIDDM, non-insulin-dependent diabetes mellitus; IL-6, interleukin-6; IL-8, interleukin-8; MCP-1, monocyte chemoattractant protein-1; sVCAM-1, soluble vascular cellular adhesion molecule; sICAM-1, soluble intracellular adhesion molecule.

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Regensburg Medical Center. Study and preparation protocol were approved by the local human ethics committee (No. 99/133).

HSVEC were isolated and cultured by a modified method according to Jaffe et al. [14]. Briefly, delivering vessels from blood components, EC were detached by incubation with 0.05% collagenase A (Roche, Basel, Switzerland), washed several times with 10 mM Hepes (Sigma, St. Louis, Missouri, USA), and cultivated in endothelial growth medium (EGM-kit; Cat.Nr. C-22010, Promocell, Heidelberg, Germany) containing 30% pooled human serum (CMS, cell culture medium with serum). Confluent cultures were dissociated and transferred to 0.1% gelatine-coated tissue culture surfaces (Merck, Darmstadt, Germany). EC stock cultures were stored in liquid $N_2$ (in CMS containing 10% DMSO) until usage. EC were characterized by their cobblestone structure monitored by phase contrast microscopy (Leica DMRBE, Bensheim, Germany) and by flow cytometric analysis using anti-human CD31 antibody staining.

*Experimental set-up.* HSVEC in passage 3 (4000 cells/cm$^2$) were seeded in 96-well microtiter-plates with 150 µl CMS and cultured under standard culture conditions. On day 3 the medium was renewed for an additional 3 days.

For growth kinetic analysis, cell counts were determined (in quadruplicate) before, 24, 48 and 72 h after medium exchange using CASY1 (Schärfe Systems, Reutlingen, Germany). For each time point, supernatant was removed, adherent cells were washed with prewarmed PBS and detached with 50 µl collagenase for 5 min at 37 °C. After addition of 100 µl CMS on ice an aliquot of the cell suspension was used to determine cell count per well.

Cellular surface expression of adhesion molecules (E-selectin, VCAM-1, ICAM-1) was detected using cellular ELISA as described in our previous study [15]. In short, at each time point during exponential (24 and 48 h) and stationary growth kinetic (72 h) EC were fixed with acetone-methanol (1:1; −20 °C, 10 min), thawed and rehydrated with PBS. For celluar ELISA, EC were blocked with 1% BSA for one hour and incubated with mouse monoclonal anti-human ICAM-1 and VCAM-1 (DAKO, Glostrup, Denmark), and anti-human E-selectin (Ancell, Bayport, MN, USA) antibodies (diluted 1:1000 in PBS; 1 h, 37 °C). Biotinylated anti-mouse antibody (Vector Laboratories, Peterborough, UK) was diluted 1:1000 for ICAM-1 and VCAM-1, 1:500 for E-selectin, and incubated for another 30 min at 37 °C. Alkaline phosphatase streptavidine (Vector) was added as a conjugate (diluted 1:1000; 30 min, 37 °C). After each incubation step cells were washed twice with PBS. After addition of *p*-nitrophenylphosphate (Sigma; 1 mg/mL in 0.1 M diethanolamine, pH 10) the absorption at 405 nm was measured with microplate-reader (Molecular Devices, Union City, CA, USA). At each time point the cellular absorption was defined as the ratio of the absorption per well and the respective cell count per well (see above).

The quantitative detection of soluble ICAM-1, VCAM-1, E-selectin and IL-6, IL-8 and MCP-1 in HSVEC cultured supernatants was performed using commercially available ELISA-kits (Biosource, Nivelles, Belgium; R&D Systems, Wiesbaden; Beckman-Coulter, Krefeld; PromoCell, Heidelberg; Germany), according to the manufacturer's instructions.

*Statistical analysis.* Data are represented as median including 25% and 75% percentile. Mann–Whitney Rank Sum Test was used to statistically verify differences between both cell groups. A *p*-value $\leqslant 0.05$ was considered to be statistically significant.

## Results

### *Cell donors and growth of isolated EC*

Demographic data of both groups of cell donors were comparable (non-diabetic patients: 1 female/7 male, $67 \pm 8$ years, high $170 \pm 7$ cm, weight $84 \pm 13$ kg, BMI $28.0 \pm 1.9$ kg/m$^2$; diabetic patients: 2 female/6 male, $64 \pm 10$ years, high $173 \pm 7$ cm, weight $77 \pm 14$ kg, BMI $27.7 \pm 3.7$ kg/m$^2$). Diabetic patients were adjusted to a mean HbA1 c of $6.3 \pm 1.4$%. No differences with respect to hemodynamics and number of risk factors (excluding diabetes mellitus) for coronary artery disease were observed between both groups of cell donors. HSVEC from normal and diabetic adults were isolated and successfully maintained in culture. The content of contaminating fibroblasts or smooth muscle cells was <1%. For immunohistological evaluation, HSVEC of both sources were characterized by their specific cobblestone-like morphology, incorporation of Dil-Ac-LDL, and positive staining for CD31 and von Willebrand Factor.

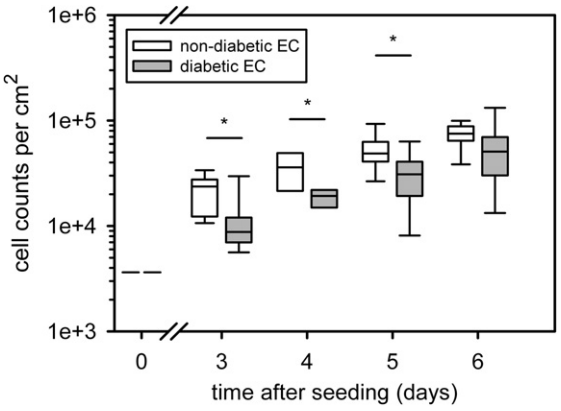


Fig. 1. Growth curves of non-diabetic and diabetic HSVEC. EC ($n = 8$ of each cell type) were seeded with a density of 4000 cells/cm$^2$ in 96-well microtiter plates and cultured for 6 days under identical culture conditions. Differences in the cell density were analyzed on day 3 to 6 ($^*p \leqslant 0.05$).

For growth kinetic analysis, EC of both groups were seeded with a density of 4000 cells/cm$^2$ and cultured under identical culture conditions for 6 days. Growth curves are shown in Fig. 1. After 3 days in culture, cell counts of non-diabetic and diabetic EC increased by a factor of 6.5 (3.4/7.3) ($p \leqslant 0.001$) and 2.4 (1.9/3.1) ($p \leqslant 0.001$), respectively. The reduced increase in cell density of diabetic cells differed significantly from the other cell group ($p \leqslant 0.05$). During the next 24 h, the number of cells increased by a factor of 2.0 (1.8/2.4) and 2.1 (2.0/2.3) for non-diabetic and diabetic cells, respectively. The doubling was significant ($p \leqslant 0.05$). The difference in cell density between non-diabetic and diabetic cells was maintained from day 3 to 5. After 6 days in culture, both cell groups reached confluence with cell densities of 75,200 (66,300/85,900) and 50,800 (42,700/61,000) EC per cm$^2$ (not significant) for non-diabetic and diabetic EC, respectively.

### *Expression of adhesion molecules*

To describe the basic state of both cell groups, we analyzed the cellular expression of surface-bound adhesion molecules under non-stimulating conditions during cell growth and in the steady state of a confluent monolayer (day 6). The basal expression of adhesion molecules of non-diabetic EC was not affected over 6 days (Fig. 2). In contrast, diabetic EC showed a growth-dependent increase