E-selectin-inducing activity in plasma from type 2 diabetic patients with maculopathy

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Knudsen, S. T., C. H. Foss, P. L. Poulsen, T. Bek, T. Ledet, C. E. Mogensen, and L. M. Rasmussen. E-selectin-inducing activity in plasma from type 2 diabetic patients with maculopathy. Am J Physiol Endocrinol Metab 284: E1–E6, 2003. First published August 13, 2002; 10.1152/ajpendo.00198.2002.—Diabetic maculopathy (DMa) is a leading cause of visual loss in the western world. We examined whether plasma from type 2 diabetic patients with DMa contains factor(s) capable of inducing expression of the adhesion molecules E-selectin and VCAM-1 or cellular proliferation in cultured endothelial cells. Four gender-, age-, and duration (diabetes groups)-matched groups of 20 subjects each participated: 1) subjects with normal glucose tolerance (NGT), 2) subjects with impaired glucose tolerance (IGT), 3) type 2 diabetic patients without retinopathy, and 4) type 2 diabetic patients with DMa. Fasting plasma was added to in vitro-grown human umbilical vein endothelial cells for 6 h, after which E-selectin and VCAM-1 expression was measured. Proliferation was evaluated by thymidine incorporation. The individuals were characterized by measurement of 24-h ambulatory blood pressure, urinary albumin excretion rate, Hb A1c, and blood lipids. Plasma from type 2 diabetic patients with DMa induced a significantly higher expression of E-selectin in endothelial cells than did plasma from subjects with NGT (259 ± 23 × 10⁻³ vs. 198 ± 19 × 10⁻³; arbitrary absorbance units; P < 0.05). There were no significant differences in plasma stimulatory effects on VCAM-1 expression or on thymidine incorporation between groups. These findings suggest that plasma from type 2 diabetic patients with DMa contains factor(s) capable of inducing the expression of E-selectin in endothelial cells. Enhanced expression of E-selectin may contribute to the development of DMa in type 2 diabetes.

Diabetic retinopathy; macular edema; diabetic nephropathy; adhesion molecules; endothelial dysfunction

Diabetic retinopathy is a leading cause of vision loss in the western world (1). Diabetic maculopathy (DMa) is the most prevalent sight-threatening manifestation of retinopathy in type 2 diabetes. Even though hyperglycemia and hypertension have been pointed out as important risk factors for the development of diabetic retinopathy (30), the basic pathogenic mechanisms that initiate this eye disease are not entirely clarified.

The development of endothelial dysfunction is considered an important element of diabetic microvascular disease. Endothelial dysfunction as seen in diabetes is associated with altered production of leucocyte-adhesive molecules, e.g., E-selectin and vascular cell adhesion molecule 1 (VCAM-1). These molecules are produced by the activated endothelium, and they are prerequisites for the adhesion and extravasation of leukocytes (13). In addition, it has been suggested that E-selectin, and maybe also VCAM-1, are involved in the angiogenic process (18). Several studies have found elevated circulating levels of the soluble, shedded part of both VCAM-1 and E-selectin in diabetes (2, 3, 12, 20), particularly among patients with microvascular disease (7, 33). Moreover, an increased expression of these molecules has been found both in the arterial wall (25, 26) and in the glomeruli (11) in diabetes. Whether increased production of VCAM-1 and E-selectin is seen in the retinal vessels in diabetes is not known at present. However, altered endothelial leukocyte adhesivity has been implied during the development of retinal lesions in diabetes (29), and data from a recent report suggest that increased expression of endothelial adhesion molecules may be involved (23).

Some clinical studies in diabetic patients have reported associations between markers of endothelial activation and hyperglycemia (4, 15, 17, 27), as well as other factors such as age, smoking, obesity, blood pressure, and blood lipids. Likewise, several experimental...
studies have found that hyperglycemia (19), glycated proteins (28), and cytokines (9) can induce endothelial cell activation in vitro. However, the causative factors behind the development of endothelial dysfunction, and in particular altered adhesion molecule expression in diabetes, are not yet known.

The aim of the present study was to examine whether plasma from 1) subjects with normal glucose tolerance (NGT), 2) subjects with impaired glucose tolerance (IGT), 3) type 2 diabetic patients without retinopathy, and 4) type 2 diabetic patients with DMa contains factors capable of stimulating the expression of adhesion molecules and the proliferation rate of cultured human endothelial cells.

MATERIALS AND METHODS

Subjects

Diabetic groups. Diabetes was diagnosed according to World Health Organization (WHO) criteria (32). Patients were considered to have type 2 diabetes if they had an onset of diabetes after the age of 30 yr, no need for insulin treatment for ≥1 yr after the diagnosis of diabetes, and no history of ketoacidosis. Twenty type 2 diabetic patients with DMa, defined as retinal hemorrhages and/or microaneurysms combined with hard exudates and/or retinal edema in the macular area, were identified in the database of eye examinations in our screening clinic for diabetic retinopathy. This database contains clinical information and fundus photographs of more than 6,000 diabetic patients from Aarhus County. Twenty type 2 diabetic patients with no sign of diabetic retinopathy were included as follows. For each patient in the maculopathy group, we identified and ranked those patients in the database with no retinopathy who matched best with regard to gender, age, and known duration of diabetes. The patients were invited to participate in the study according to rank.

Control subjects. Control subjects were recruited from the Fredericia Study (8). For each patient in the DMa group, we identified and included a control subject with normal glucose tolerance (NGT) as well as a subject with impaired glucose tolerance (IGT) who matched best with regard to gender and age. The classification of subjects having NGT or IGT was based on the result of an oral glucose tolerance test (OGTT), which was evaluated according to WHO criteria (32). All subjects included in the study were Caucasians, and all gave their written informed consent to participate. The study was approved by the regional ethics committee.

Methods

Eye examinations. All diabetic patients underwent a routine ophthalmological examination for diabetic retinopathy, including measurement of visual acuity, slit lamp examination, fundus photography, and fluorescein angiography. Fundus photography was performed using a Canon 60UV fundus camera on Kodak Ectachrome 64 color diapositive film. In each eye, one 60° image centered on the fovea and one nasally displaced field centered on the optic disk were taken. Fluorescein angiography was performed on Ilford Delta 400 black/white film. A fast sequence was taken during the filling phase of the retinal vessels on the study eye, and late-phase images were taken on both eyes 5–10 min after the injection of fluorescein. On the basis of the fluorescein angiograms, it was verified that all maculopaties were of an exudative type.

Clinical biochemical analyses. Urinary albumin excretion rate (UAE) was measured by RIA and expressed as the geometric mean of three overnight collections made within 1 wk. Hb Alc was determined by HPLC (nondiabetic range 4.4–6.4%). Blood glucose was determined by Replifux II, Boehringer Mannheim.

Twenty-four-hour ambulatory blood pressure measurements. Twenty-four-hour ambulatory blood pressure (AMBP) was measured by an oscillometric technique [Spacelabs 90207, Redmond, WA, validated by the British Hypertension Society (21)]. Readings were obtained at 20-min intervals throughout 24 h. Measurements were performed during a day with normal activities at home or at work. Individually reported sleeping times were implemented in the calculation of day and night BP.

Patients were classified as nonsmokers (without daily use of tobacco for the preceding year) or smokers.

Plasma samples. EDTA-plasma samples for cell culture testing were collected after an overnight fast (10–12 h).

Cell Culture Experiments

Cell cultures of human umbilical vein endothelial cells. Human umbilical vein endothelial cells (HUVEC) obtained from collagenase-digested umbilical veins were cultured in Dulbecco's modified Eagle medium (DMEM; glucose concentration 5.5 mM) containing 10% fetal calf serum (FCS), 2 μg/ml ciprofloxacin, 100 μg/ml ampicillin, 1% L-glutamine, 40 μg/ml endothelial cell growth factor (ECGF), 15 U/ml heparin, and 5 mM glutamine in gelatin-coated plates (0.65 g/ml endothelial cell growth factor (ECGF), 15 U/ml heparin, and 5 mM glutamine in gelatin-coated plates (0.65 g/ml) and maintained at 37°C in an atmosphere of 5% CO2–95% atmospheric air. The cells were subcultured after detachment with trypsin solution and replating. Cells were used between the 3rd and 6th trypsinization. Cell counting was performed after trypsinization by use of a Bürker-Türk chamber. Viability of cells was evaluated by morphology and trypan blue exclusion.

ELISA procedures for cellular content of VCAM-1 and E-selectin. A modified ELISA procedure was used to measure the cellular E-selectin and VCAM-1 content (24). Cells were grown in 96-well plates, exposed to 10% test plasma in normal medium for 6 h, subsequently washed once with 150 μl of PBS, fixed in 150 μl of 100% methanol for 10 min, air-dried, and stored at 4°C. Dried cells were rehydrated and blocked in 150 μl of PBS, 0.1% Tween 20, 0.5% BSA (P + T + A) for 30 min and washed twice in P + T. The wells were then incubated for 2 h at room temperature with either a monoclonal antibody against human E-selectin (BBA-16, R&D Systems) diluted 1:500 in P + T + A or a polyclonal goat antibody against human VCAM-1 (BBA-19, R&D Systems) diluted 1:500 in P + T + A. After two washes in P + T, wells were incubated with horseradish peroxidase (HRP)-conjugated secondary antibodies diluted in P + T + A: rabbit anti-mouse Ig-HRP (NA9310, Amersham Life Sciences) 1:4,000 for E-selectin measurements and rabbit anti-goat Ig-HRP (P0160; Dako, Copenhagen, Denmark) 1:4,000 for VCAM-1 analysis. After 1 h incubation at room temperature, wells were washed five times in P + T, and they were subsequently colored using 100 μl of TMB-reagent (Dako S 1800) as substrate for the bound HRP. After 5 min of incubation, the reaction was stopped by adding 100 μl of 3 M H2SO4. Absorbance was read at 540 nm in an ELISA reader. Every individual plasma sample was analyzed in quadruplicate, and a mean value was calculated. In all, 640 cultures were used for the analysis of adhesion molecules.
Statistical Analysis

All parameters were tested for normal distribution by use of the Kolmogorov-Smirnov nonparametric test, and parameters nonnormally distributed were log-transformed before statistical analysis. Differences between groups were tested by the Student's t-test (unpaired). For noncontinuous variables, the χ² test with Yates's correction was used. Correlations were analyzed using Pearson's test. A two-tailed P value of <0.05 was considered significant. Results for normally distributed variables are expressed as means ± SE, whereas UAE values are expressed as the geometric mean ×/−tolerance factor.

RESULTS

Clinical and laboratory characteristics of study participants are presented in Table 2. The expression of E-selectin in endothelial cells was lowest in the NGT group and increased in a stepwise manner over the IGT and no-retinopathy groups to the DMa group (Fig. 1), where the mean E-selectin content of endothelial cells was significantly higher than in the NGT group (258 ± 23 × 10<sup>3</sup> vs. 198 ± 19 × 10<sup>3</sup> arbitrary absorbance units, P < 0.05). The expression of E-selectin in endothelial cells induced by plasma from all diabetic patients (n = 40) was significantly higher than the expression induced by plasma from the subjects in the NGT group (248 ± 15 × 10<sup>3</sup> vs. 198 ± 19 × 10<sup>3</sup> arbitrary absorbance units, P < 0.05), whereas there was no significant difference in this parameter between the diabetic and IGT groups. There were no significant differences in the expression of VCAM-1 or thymidine incorporation between the groups (Table 2).

No correlations between the expression of E-selectin and VCAM-1 or thymidine incorporation of endothelial cells after addition of plasma and Hb A₁c or fasting blood glucose were observed. Likewise, the plasma-induced thymidine incorporation and expression of E-selectin and VCAM-1 by endothelial cells did not correlate with age, gender, known diabetes duration, smoking, antidiabetic treatment modality, BMI, BP measures, blood lipids, or UAE.

DISCUSSION

The main finding of the present study was the increased ability of plasma from patients with type 2 diabetes to induce the expression of E-selectin in endothelial cells. The effect on E-selectin expression was lowest in plasma from normal subjects, increased in a stepwise manner in the groups with impaired glucose tolerance and uncomplicated type 2 diabetes, and reached a maximum in the group with DMa. In con-
contrast, we could not demonstrate any differences in the effect on VCAM-1 expression. Likewise, we found no difference in thymidine incorporation among the groups. Although increased division of endothelial cells may be involved in the development of proliferative retinopathy, this finding is compatible with the fact that DMa is not characterized by excessive cell proliferation.

Several studies have demonstrated an increased level of soluble adhesion molecules in plasma of diabetic patients, indicating an enhanced expression of these molecules on endothelial cells in diabetes (2, 3, 12, 20). The results of the present study expand these findings, suggesting that one or more circulating factors stimulate the expression of E-selectin on the endothelial surface in type 2 diabetes. Previously, an association between increased levels of circulating adhesion molecules and the presence of diabetic complications has been described (7, 33). In the present study, we found that the stimulatory effect on E-selectin in endothelial cells tended to be more pronounced in patients with DMa than in those without retinopathy. This observation supports the idea of an association between enhanced endothelial activation and the presence of DMa.

Increased levels of adhesion molecules have been found in the vitreous of patients with proliferative diabetic retinopathy (10, 14). However, only a few studies have examined the connection between expression of endothelial adhesion molecules and the development and progression of diabetic retinopathy. In type 1 diabetic patients, Olson et al. (23) found a highly significant association between the degree of retinopathy and the amount of circulating E-selectin and VCAM-1. In the same study, Olson et al. demonstrated that sera from patients with retinopathy contain factor(s) capable of inducing increased migratory activity when added to endothelial cells in vitro. Furthermore, their data indicated that these circulating factors may at least partly be the soluble forms of both VCAM-1 and E-selectin. Our results are in line with the observations by Olson et al. We found the highest E-selectin-inducing activity in plasma from patients with DMa, comparable to the groups with early, nonproliferative stages of retinopathy, in which the highest E-selectin values were detected in the study by Olson et al. In contrast, we found no difference in VCAM-1-inducing activity between groups, still in line with the findings from Olson et al., where the highest VCAM-1 concentrations were seen in patients with proliferative retinopathy, a group that was not included in our study. From a combination of the current data and the results from the report of Olson et al., it may be hypothesized that increased E-selectin expression is present in patients with early stages of retinopathy, whereas VCAM-1 expression may dominate in the proliferative stages. Moreover, the data of Olsen et al. seem to suggest that increased expression of adhesion molecules is involved in the transition from nonproliferative to proliferative retinopathy. Our data, on the other hand, point toward the presence of circulating factor(s) capable of inducing increased expression of E-selectin in the earlier stages of diabetic retinopathy. Caution should, however, be observed when interpretations are made from these in vitro data.

In type 1 diabetes, hyperglycemia per se is the single most obvious factor promoting enhanced endothelial activation, whereas the possible candidates in type 2 diabetes are numerous: aging, hypertension, hyperglycemia, insulin resistance, and dyslipidemia, to mention a few (5). Many previous studies on this subject have been hampered by confounding factors such as age, sex, and duration of diabetes. In the present study, we eliminated such confounding factors by closely matching the individuals from each group on these parameters. We found no correlation between glycemic control and the effect of plasma on the expression of E-selectin in endothelial cells. Likewise, no association between

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**Table 2. Effect of plasma from different study groups on content of E-selectin and VCAM-1 and on thymidine incorporation in endothelial cells**

<table>
<thead>
<tr>
<th></th>
<th>Control Groups</th>
<th>Type 2 Diabetes</th>
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<tr>
<td></td>
<td>NGT</td>
<td>IGT</td>
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<tr>
<td>E-selectin (aa units × 10^3)</td>
<td>198 ± 19</td>
<td>225 ± 22</td>
</tr>
<tr>
<td>VCAM-1 (aa units × 10^3)</td>
<td>856 ± 35</td>
<td>854 ± 33</td>
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<tr>
<td>Thymidine, cpm/well</td>
<td>2,326 ± 81</td>
<td>2,298 ± 109</td>
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Values are means ± SE. VCAM-1, vascular cell adhesion molecule; aa, arbitrary absorbance. *P < 0.05 vs. NGT.

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**Fig. 1. Induction of E-selectin in endothelial cells by plasma from individuals with normal glucose tolerance (NGT) and impaired glucose tolerance (IGT) and from diabetic patients with and without maculopathy, as indicated. Plasma from each individual was incubated for 6 h, and E-selectin amount was measured. Values are expressed as means ± SE (n = 20 for each group).** *P < 0.05.*
the expression of E-selectin and other factors like age, diabetes duration, gender, BMI, lipids, BP, or UAE could be demonstrated. However, some of these factors may still be of importance for the development of endothelial dysfunction in vivo, as the design of the present in vitro experiment allows only the detection of effects mediated by humoral substances. An obvious example is hypertension, which may promote endothelial activation through hemodynamic mechanisms, e.g., cyclic stretch of endothelial cells (31). Nevertheless, our findings suggest that circulating factors do indeed have a role.

Morigi et al. (17) found an increased monocyte adhesion capacity of human endothelial cells when exposed to serum from diabetic patients, as well as to high glucose concentrations. We have recently performed a series of experiments in which alterations in the glucose concentrations. We have recently performed a series of experiments in which alterations in the glucose concentration of the growth medium (5.5–13.5 mM) did not change the expression of either VCAM-1 or E-selectin in human endothelial cells (24a). Likewise, we found no correlation between the level of glycemia and the expression of E-selectin and VCAM-1 in endothelial cells in the present study, whereas results from previous studies have been conflicting (4, 6, 7, 15, 16, 27, 33).

In a recent study, high insulin concentrations have been shown to enhance neutrophil-endothelial cell adhesion in vitro (22). However, the effect of insulin seemed to be mediated through an increased endothelial expression of ICAM-1, whereas there was no effect of insulin concentration on the expression of VCAM-1. In the present study, we found no association between antidiabetic treatment modality (e.g., insulin dose) and endothelial cell expression of VCAM-1 and E-selectin.

Several other plasma constituents, e.g., advanced glycation end products (28), tumor necrosis factor-α (9), and hormonal factors, have been shown to alter adhesion molecule expression in vitro, and one or more of these substances may contribute to the findings of the present study. However, further studies are needed to identify the factor(s) responsible for the enhanced expression of adhesion molecules on endothelial cells in diabetes.

In conclusion, we have shown that plasma from type 2 diabetic patients with maculopathy contains factor(s) capable of inducing the expression of E-selectin in human endothelial cells. This effect cannot directly be ascribed to hyperglycemia. Enhanced expression of E-selectin may contribute to the development of maculopathy in type 2 diabetes.

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REFERENCES