Activity of the Glycosylating Enzyme, Core 2 GlcNAc (\(\beta1,6\)) Transferase, Is Higher in Polymorphonuclear Leukocytes From Diabetic Patients Compared With Age-Matched Control Subjects

Relevance to Capillary Occlusion in Diabetic Retinopathy

Rakesh Chibber, Bahaedin M. Ben-Mahmud, David Coppini, Emmanuel Christ, and Eva M. Kohner

The exact mechanism for capillary occlusion in diabetic retinopathy is still unclear, but increased leukocyte-endothelial cell adhesion has been implicated. We examined the possibility that posttranslational modification of surface O-glycans by increased activity of core 2 transferase (UDP-Glc:Gal\(\beta1\)-3GalNAc\(\beta\)-R\(\beta\)-N-acetylglucoaminyltransferase) is responsible for increased adhesion of leukocytes to vascular endothelium in diabetes. The mean activity of core 2 transferase in polymorphonuclear leukocytes isolated from type 1 and type 2 diabetic patients was higher compared with age-matched control subjects (1,638 ± 91 [\(n = 42\)] vs. 249 ± 35 pmol · h\(^{-1}\) · mg\(^{-1}\) protein [\(n = 24\)], \(P = 0.00013\); 1,459 ± 194 [\(n = 58\)] vs. 334 ± 86 [\(n = 11\)], \(P = 0.01\)). As a group, diabetic patients with retinopathy had significantly higher mean activity of core 2 transferase compared with individuals with no retinopathy. There was a significant association between enzyme activity and severity of retinopathy in type 1 and type 2 diabetic patients. There was a strong correlation between activity of core 2 transferase and extent of leukocyte adhesion to cultured retinal capillary endothelial cells for diabetic patients but not for age-matched control subjects. Results from transfection experiments using human myelocytic cell line (U937) demonstrated a direct relationship between increased activity of core 2 transferase and increased binding to cultured endothelial cells. There was no relationship between activity of core 2 transferase and HbA\(_1c\) (\(P = 0.8314\)), serum advanced glycation end product levels (\(P = 0.4159\)), age of the patient (\(P = 0.7896\)), and duration of diabetes (\(P = 0.3307\)). On the basis that branched O-glycans formed by the action of core 2 transferase participate in leukocyte adhesion, the present data suggest the involvement of this enzyme in increased leukocyte-endothelial cell adhesion and the pathogenesis of capillary occlusion in diabetic retinopathy. Diabetes 49:1724–1730, 2000

Diabetic retinopathy is a progressive vision-threatening complication of diabetes (1) characterized by capillary occlusion, formation of microvascular lesions, and retinal neovascularization adjacent to ischemic areas of the retina (2,3). The exact pathogenic mechanism by which capillary nonperfusion as demonstrated by fluorescein angiography occurs is still unclear, but studies in the last decade have suggested that leukocytes may play an important role in its development (4–9). The white cells are relatively large with high cytoplasmic rigidity (10), often completely filling the capillary lumen; thus, significantly higher forces are needed to deform them during their passage through the microvessels. In vitro studies have shown that monocytes isolated from diabetic patients are more adhesive to cultured human endothelial cells than those from healthy control subjects and that this leukocyte-endothelial cell adhesion is CD11-CD18 dependent (11). More recently, Morigi et al. (12) have demonstrated that acute exposure of cultured human endothelial cells to high glucose concentration promotes leukocyte-endothelial cell adhesion by upregulating cell surface expression of adhesive proteins through the nuclear factor-\(\kappa\)B and protein kinase C (PKC) intracellular pathways.

There is also compelling evidence for the involvement of leukocytes in diabetic retinopathy from studies using human tissue that demonstrate a strong relationship between leukocyte-endothelial cell adhesion and retinal capillary damage in diabetes (13). Leukocytes isolated from type 2 diabetic patients have decreased deformability and thus the potential to damage retinal capillaries (4). In animal models of diabetes,
enhanced superoxide radical production by stimulated leucocytes and increased leucocyte accumulation in the endothelium has been demonstrated (14,15). Schröder et al. (16) demonstrated that leucocytes can cause capillary occlusion in diabetic rats, resulting in areas of capillary nonperfusion and destruction of the capillary bed. More recently, Miyamoto et al. (17) have shown increased leucocyte entrapment in retinal capillaries of diabetic rats. In addition to the retina, leucocyte plugging has also been reported to increase throughout the capillary network in the skeletal muscle of the diabetic rat (18).

Leucocytes adhere to the vascular endothelium through selectins (19) and subsequently through adhesion molecules such as integrins (20). O-linked oligosaccharides (glycoproteins/glycolipids) on the surface of leucocytes play a crucial role in leucocyte–endothelial cell adherence through selectins and integrins (19,20). In this study, we explored the underlying mechanism that may be responsible for increased leucocyte-endothelium adhesion in diabetes. We proposed that raising activity of the key glycosylating enzyme, core 2 GlcNAc-T (UDP-Glc:Galβ1-3GalNAcα-R β-N-acetylglucaminyltransferase), may lead to changes in the expression of O-linked oligosaccharides on the surface of leucocyte and increased adhesion to endothelial cells. Core 2 transferase is a Golgi enzyme that substitutes core 1 O-linked glycans (i.e., Galβ1,3GalNAcα) to produce core 2 structures (i.e., Galβ3(GlcNAcβ1–6)GalNAcα). Core 2 transferase appears to be an important rate-limiting step in the extension of O-linked oligosaccharides, and its activity may be regulated by factors that have an impact on intracellular signaling and development status of the cells (21). Recently, Nishio et al. (22) have demonstrated that hyperglycemia and insulin can also modulate the activity of core 2 transferase in the hearts of diabetic rats and in cultured cardiomyocytes.

**RESEARCH DESIGN AND METHODS**

**Subjects.** This study included 42 type 1 and 60 type 2 diabetic patients recruited from the Diabetes Outpatient Clinic and the Eye Unit at St. Thomas’ Hospital. For characteristics of type 1 and type 2 diabetic patients, see Tables 1 and 2, respectively. The healthy control subjects were recruited from family members and friends accompanying the patients to the clinic or from hospital employees. The severity of retinopathy was graded according to the Early Treatment Diabetic Retinopathy Study (ETDRS) levels of 20–47, which indicated mild and moderate nonproliferative retinopathy and macular edema, and levels of 33 and higher, which indicated severe nonproliferative retinopathy, proliferative retinopathy, and advanced eye disease.

**Collection of blood and isolation of polymorphonuclear leucocytes.** Whole blood was drawn and collected in heparinised tubes. Pure polymorphonuclear leucocyte (PMN) suspensions were prepared by density gradient centrifugation over Ficoll-Paque (Pharmacia). In brief, 10 ml whole blood was layered onto an equal volume of Ficoll-Paque and centrifuged (Heraeus, Brentwood, Essex, U.K.) at 400g for 30 min. The PMN-rich buffy coat was carefully removed, resuspended in phosphate-buffered saline (PBS), centrifuged at 400g for 15 min, and the pellet stored at –20°C until used in the assay for core 2 transferase.

**Analytical methods.** Analyses of plasma glucose and HbA1c levels were carried out by standard laboratory techniques. Serum levels of advanced glycation end products (AGEs) were determined as previously described (23) by enzyme-linked immunosorbant assay (ELISA) using a specific polyclonal antibody. Standard curve for the measurement of AGE was obtained using AGE-modified bovine serum albumin (BSA) prepared by incubating BSA with glucose for 60 days (23). The optical density (OD) of the samples was read at 490 nm (Shimadzu, Kyoto, Japan) and the amount of AGE in the serum calculated as B/Bo where B = OD(competition) – background OD(no antibody) and Bo = total OD(no competition) – background OD(no antibody). One unit of AGE was defined as the amount of antibody-reactive AGE that was equivalent to 1 nmol of the AGE-modified BSA.

<table>
<thead>
<tr>
<th>TABLE 1</th>
<th>Clinical characteristics of type I diabetic patients</th>
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<td>Normal control subjects</td>
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<td>Age (years)</td>
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<td>Duration of diabetes (years)</td>
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<tr>
<td>Sex (F/M)</td>
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<td>HbA1C (%)</td>
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Data are n, means ± SD, or %

**Measurement of ([β1-6]GlcNAc transferase (core 2 transferase) enzyme activity.** Leucocytes were washed in PBS, frozen, and lysed in 0.9% Triton X-100 at 0°C. The activity of core 2 transferase was measured as described previously (24). Briefly, the reaction was performed in a reaction mixture containing the following: 50 mmol/l 2(N-morpholine) 2(N-morpholino) ethanesulfonic acid (Sigma, Dorset, U.K.), pH 7.0; 1 mmol/l UDP-[6-3H]-N-acetylglucosamine (16,000 dpm/mmol, NEN Life Science Products, Hounslow, U.K.); 0.1 mol/l GlcNAc (Sigma); 1 mmol/l βDgal (1-3)-Dxy-GalNAc-p-nitrophenoL (Sigma) as substrate; and 16 μl cell lysate (100–200 μg protein) for a final volume of 32 μl. After incubating the mixture for 1 h at 37°C, the reaction was terminated with 1 ml ice-cold water and processed on a C18 Sep-Pak column (Waters-Millipore, Watford, U.K.). After washing the column with 20 ml water, the product was eluted with 5 ml methanol. The radioactivity of the samples was counted in a liquid scintillation β-counter (LKB-Wallac, London, U.K.). Endogenous activity of core 2 transferase was measured in the absence of the added acceptor. The specific activity was expressed as picomoles per hour per milligram of cell protein (24).

In each case, the protein concentration was determined with protein assay (BioRad, Hertfordshire, U.K.).

**Culture of bovine retinal capillary endothelial cells.** Primary and subculture (up to the second passage) of bovine retinal capillary endothelial cells (BRECs) was established from bovine retinas dissected from eyes of freshly slaughtered cattle as described previously (25). Briefly, the isolated retinas were homogenized in serum-free minimal essential medium (MEM) (Gibco, Paisley, U.K.) and filtered through 85-μm nylon mesh. The trapped microvessels were digested with collagenase-dispase (1 mg/ml) for 90 min at 37°C and filtered through a 53-μm nylon mesh. The digested microvessels were then plated in gelatin-coated tissue culture flasks. The growth medium for BRECs was MEM supplemented with 10% pooled human serum. The cells were characterized using morphological criteria and by immunostaining with an antibody against factor VIII–related antigen.

**TABLE 2**

<table>
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<td>Sex (F/M)</td>
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<tr>
<td>HbA1C (%)</td>
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<td>With no retinopathy (n)</td>
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<tr>
<td>With background retinopathy (n)</td>
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<tr>
<td>With preproliferative and proliferative retinopathy (n)</td>
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<tr>
<td>With maculopathy (n)</td>
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</table>

Data are n, means ± SD, or %
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Culture of human myelocytic cell line (U937). U937 cells were cultured in RPMI with 10% fetal calf serum.

Isolation of core 2 cDNA and transfection experiments. Core 2 cDNA that was isolated from a previously described HL60 cDNA library (26) was provided by Dr. Kumar (Small Molecule Drug Discovery, Genetics Institute, Cambridge, MA). The clone pMTCore 2, containing an insert of ~2.0 kb, has been previously sequenced and found to be identical to the published sequence (27). Plasmid DNA (pMTCore2) for the transfection experiments was prepared using a plasmid purification system (Qiagen, West Sussex, U.K.).

Parental U937 cells were transfected with core 2 cDNA (pMTCore 2) using the Transfast transfection kit (Promega) according to the suggested protocol. The transfected cells were selected in medium containing 1 mg/ml G418 (Sigma), and the stable clone U937 + Core 2 was isolated.

Leukocyte-endothelial cell adhesion assay. The aim of this part of the study was to examine whether increased activity of core 2 transferase in leukocytes from diabetic patients is associated with increased adhesion of the leukocytes to cultured retinal capillary endothelial cells. The leukocyte-endothelial cell adhesion assay was carried out as described previously (28). Briefly, endothelial cells were grown in chamber slides to a confluent state, thus providing an endothelial cell surface for the adhesion of leukocytes. PMNs were isolated and added to tissue culture dishes to remove adherent monocytes. After 30 min at 37°C, PMNs were removed from the flasks, counted by means of a hemocytometer, and added to the endothelial cell monolayer. After 30 min at 37°C, the dishes were fixed in 3.7% formalin in PBS and the number of adherent leukocytes counted in 10 random high-powered fields (Olympus, London).

The results were expressed as the percentage of adherent leukocytes per field.

Statistical analysis. The statistical software Graphpad Prism version 3.0 was used. The Student’s t test was used to test the significance of variables between the diabetic patients and age-matched control subjects. Linear regression and correlation were used to evaluate the relationship between two variables. P < 0.05 was considered statistically significant. All results are given as means ± SE.

Results

Activity of core 2 transferase in leukocytes from diabetic patients and control subjects. The activity of core 2 transferase was found to be higher in type 1 diabetic patients compared with age-matched control subjects (Fig. 1) with the mean activity almost threefold higher (1,638 ± 291 [n = 42] vs. 249 ± 35 pmol · h⁻¹ · mg⁻¹ protein [n = 25], P = 0.00013). There was a significant difference in enzyme activity between type 1 diabetic patients with and without retinopathy (2,096 ± 409 [n = 29] vs. 444 ± 154 [n = 12] pmol · h⁻¹ · mg⁻¹ protein, P = 0.00031). A similar trend in the activity of core 2 transferase was also found for type 2 diabetic patients. The activity of core 2 transferase was almost threefold higher in leukocytes from type 2 diabetic patients compared with healthy control subjects (1,459 ± 194 [n = 60] vs. 334 ± 86 pmol · h⁻¹ · mg⁻¹ protein [n = 11], P = 0.01). There was a significant difference in enzyme activity between type 2 diabetic patients with and without retinopathy (1,798 ± 248 [n = 39] vs. 551 ± 124 [n = 21] pmol · h⁻¹ · mg⁻¹ protein, P = 0.00003).

Leukocytes isolated from diabetic patients with no retinopathy had significantly higher activity of core 2 transferase compared with those from healthy control subjects (512 ± 92 [n = 25] vs. 283 ± 39 [n = 31] pmol · h⁻¹ · mg⁻¹ protein, P = 0.0187). As shown in Fig. 2, worsening of retinopathy was associated with a significant increase in the activity of core 2 transferase in leukocytes of diabetic patients. Compared with diabetic patients with no retinopathy, the mean activity in diabetic patients with mild and moderate nonproliferative retinopathy was almost threefold higher (1,444 ± 228 [n = 43] vs. 512 ± 92 pmol · h⁻¹ · mg⁻¹ protein [n = 25], P < 0.0001). Diabetic patients with proliferative retinopathy had significantly higher activity compared with those with mild and moderate nonproliferative retinopathy (2,219 ± 307 [n = 27] vs. 1,444 ± 228 pmol · h⁻¹ · mg⁻¹ protein, [n = 43], P = 0.043). Leukocytes of diabetic patients with maculopathy had almost threefold higher activity compared with those from
patients with no retinopathy ($1,748 \pm 535$ [n = 11] vs. $283 \pm 39$ pmoles/h/mg protein [n = 31], $P = 0.0023$).

**Relationship between the activity of core 2 transferase, age of the patient, duration of diabetes, HbA$_{1c}$, and serum AGE levels.** The relationship between the activity of core 2 transferase and age of the patient, duration of diabetes, and HbA$_{1c}$ for type 1 and type 2 diabetic patients is presented in Fig. 3. There was no significant correlation between activity of core 2 transferase in PMNs and age of the diabetic patient ($r = 0.054$, $P = 0.625$), duration of diabetes ($r = 0.076$, $P = 0.520$), HbA$_{1c}$ levels ($r = 0.061$, $P = 0.669$), and serum AGE level ($r = 0.204$, $P = 0.4159$).

**Adhesion of PMNs to cultured retinal capillary endothelial cells.** Compared with PMNs from age-matched control subjects, the ones isolated from type 1 and type 2 diabetic patients demonstrated increased adhesion to cultured retinal capillary endothelial cells (Fig. 4). The mean level of leukocyte-endothelial cell adhesion was found to be almost 18-fold higher for PMNs isolated from diabetic patients than those from age-matched control subjects ($5.340 \pm 0.17\%$ [n = 15] vs. $0.329 \pm 0.17\%$ [n = 14], $P = 0.004$).

The relationship between the extent of PMN adhesion to endothelial cells and activity of core 2 transferase is shown in Fig. 5. For type 1 and type 2 diabetic patients, a highly significant correlation (Fig. 5B) was observed ($r = 0.52$, $P = 0.008$) between activity of core 2 transferase and extent of leukocyte-endothelial cell adhesion. In the age-matched control subjects, there was no significant correlation ($r = 0.0068$, $P = 0.9745$) between enzyme activity and extent of leukocyte-endothelial cell binding (Fig. 5A).

**Transfection of U937 cells with core 2 cDNA.** To provide direct experimental evidence for our hypothesis that increased activity of core 2 transferase leads to increased binding to endothelial cells, the cDNA for core 2 (pMT Core2) was transfected into parental U937 cells, and a new stable cell line (U937 + C2) was subsequently isolated. The lysates of the transfected (U937 + C2) cells had significantly
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FIG. 5. Relationship between activity of core 2 transferase and percent leukocyte binding to retinal capillary endothelial cells. Leukocytes isolated from healthy age-matched control subjects (A) and from diabetic patients (B) and were added to a monolayer of endothelial cells. After incubation for 30 min at 37°C, the number of adherent leukocytes was counted in 10 high-powered random fields and results expressed as percent of leukocyte binding. For type 1 and type 2 diabetic patients, there was a significant correlation between activity of core 2 transferase and percent of leukocyte binding ($r = 0.52$, $P = 0.008$). There was no significant correlation between enzyme activity and percent leukocyte binding in healthy nondiabetic control subjects ($r = 0.00082$, $P = 0.97$).

FIG. 6. Activity of core 2 transferase (A) and extent of binding to endothelial cells (B) of parental (U937) and transfected (U937 + core 2) U937 cells. Results are presented as means ± SE of five separate experiments. *$P = 0.0065$ vs. parental U937 cells; **$P < 0.0001$ vs. parental U937 cells.

increased activity of core 2 transferase ($5,018 ± 1,364 \text{ [n = 5]}$ vs. $172.4 ± 55.11 \text{ [n = 5]}$, $P = 0.0075$) and binding to ($5.134 ± 0.1927 \text{ [n = 5]}$ vs. $1.050 ± 0.1480 \text{ [n = 5]}$, $P < 0.0001$) endothelial cells compared with the parental U937 cells (Table 3).

DISCUSSION
In the present study, we have further examined the concept that leukocytes are involved in the development of vaso-occlusion/nonperfusion in diabetic retinopathy. The primary goal was to test the hypothesis that in diabetes, increased leukocyte–endothelial cell adhesion and consequent capillary obstruction are caused by posttranslational modification of O-linked oligosaccharides (O-glycans) on the surface of leukocytes.

Our results showing that leukocytes isolated from type 1 and type 2 diabetic patients have increased adherence to cultured retinal capillary endothelial cells are in agreement with previous data on monocytes. For instance, Dosquet et al. (11) have shown that monocytes from diabetic patients have increased surface expression of the adhesion molecule CD11b-CD18 and are much more adhesive to endothelial cells than those from age-matched control subjects. Exposure of neutrophils to high glucose concentrations also increases their adhesion to retinal capillary endothelial cells. Leukocytes that become trapped in capillaries may become primed to undergo proliferation within tissues and release not just angiogenic factors (29) in response to resulting hypoxia but also oxygen-derived free radicals (14–15), which can damage endothelial cells (30) and lead to the formation of acellular capillaries. The key enzyme involved in the biosynthesis and elongation of O-glycans is core 2 transferase (31). The possibility of this enzyme being involved in increased leukocyte–endothelial cell adherence in diabetes is supported by three sets of results. First, the activity of this enzyme is significantly higher in the leukocytes isolated from type 1 and type 2 diabetic patients compared with those from age-matched healthy control subjects. Second, the activity of core 2 transferase is significantly higher in leukocytes isolated from diabetic patients with retinopathy than those isolated from patients without retinopathy. Worsening of retinopathy is associated with increasing activity of core 2 transferase in leukocytes of diabetic patients. Third, there is a significant correlation between the activity of core 2 transferase and the extent to which leukocytes adhere to cultured endothelial cells. This relationship between activity of core 2 transferase
and leukocyte–endothelial cell binding was further supported by the finding that transfection of core 2 cDNA into a human myelocytic cell line (U937) increased their binding to cultured endothelial cells. Taken together, these results provide support for our postulate that diabetes may cause post-translational modification of O-glycans on the surface of leukocytes by increasing the activity of core 2 transferase. The resulting structural alteration in the O-glycans may be responsible for increased adherence of leukocytes to vascular endothelium and for the development of microvascular complications in diabetic retinopathy.

A number of O-glycans on the surface of leukocytes are thought to play an important role in leukocyte–endothelial cell binding through interaction with sialyl lectins and integrins (19,20). The selectins interact with sialylated and fucosylated lactosaminoglycans, such as sialyl Lewis x (sLex), but bind with high affinity/avidity to O-glycans displayed on a limited number of glycoproteins (32). Recent research supports a physiological role for one of these glycoproteins, P-selectin glycoprotein ligand 1 (PSGL-1). PSGL-1 accounts for all of the high-affinity binding sites to P-selectin and has many clustered sialylated O-linked glycans (33), some with polylactosamine terminations in sLex (34). It is tempting to put forward the idea that the observed increased activity of core 2 transferase in diabetic leukocytes may lead to the posttranslational modification of PSGL-1 and increased leukocyte–endothelial cell binding. Interestingly, a recent study has shown that high-affinity binding of PSGL-1 requires core 2 transferase activity (35). It will be of great significance to perform a detailed analysis of the O-glycans from leukocytes to test whether increased activity of core 2 transferase does lead to posttranslational modification of PSGL-1 and/or other glycoproteins on the surface of leukocytes from diabetic patients with and without retinopathy.

The factor or factors responsible for increased activity of core 2 transferase in diabetic PMNs are unclear, but hyperglycemia has been shown to modulate the activity in the heart of diabetic rats and in cultured cardiomyocytes, possibly through the diacylglycerol–PKC pathway (22). Our failure to find a significant relationship between HbA1c, and the activity of core 2 transferase probably suggests that hyperglycemia is not directly responsible for increased activity of this enzyme in diabetic PMNs. Because the Diabetes Control and Complications Trial (36) has confirmed that hyperglycemia is a primary factor in the development and progression of retinopathy, we suggest that capillary occlusion in diabetes results at least in part, from both surface changes on the leukocytes and hyperglycemia-induced expression of leukocyte adhesion molecules on endothelial cells. Firm adhesion of leukocyte to vascular endothelium is controlled by integrins and the expression of adhesion molecules, including intercellular cell adhesion molecule 1 (ICAM-1), vascular cell adhesion molecule, E-selectin, and P-selectin (37). In human diabetes, increased expression of ICAM-1 and P-selectin has been reported on vascular endothelial cells of pancreatic islets and in the retina and choroid (38,39). Recent in vitro studies have also shown that high glucose concentration and AGEs can induce the expression of adhesion molecules on cultured endothelial cells (40–42).

In conclusion, our results indicate the following: 1) the activity of the glycosylating enzyme, core 2 transferase, is increased in leukocytes from diabetic patients, particularly those with retinopathy; and 2) there is a significant relationship between the activity of core 2 transferase and the level of leukocyte–endothelial cell adhesion. These results support the possibility that leukocyte entrapment in diabetes may be mediated by increased expression of adhesion molecules on the surface of endothelial cells and the modification of O-linked oligosaccharides on the surface of diabetic PMNs.

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