Integrin-Mediated Neutrophil Adhesion and Retinal Leukostasis in Diabetes

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PURPOSE. A critical early event in the pathogenesis of diabetic retinopathy is leukocyte adhesion to the diabetic retinal vasculature. The process is mediated, in part, by intercellular adhesion molecule-1 (ICAM-1) and results in blood-retinal barrier breakdown and capillary nonperfusion. This study evaluated the expression and function of the corresponding ICAM-1–binding leukocyte β₂-integrins in experimental diabetes.

METHODS. Diabetes was induced in Long Evans rats with streptozotocin. The expression of the surface integrin subunits CD11a, CD11b, and CD18 on rat neutrophils isolated from peripheral blood was quantitated with flow cytometry. In vitro neutrophil adhesion was studied using quantitative endothelial cell-neutrophil adhesion assays. The adhesive role of the integrin subunits CD11a, CD11b, and CD18 was tested using specific neutralizing monoclonal antibodies. CD18 bioactivity was blocked in vivo with anti-CD18 F(ab)’2 fragments, and the effect on retinal leukocyte adhesion was quantitated with acridine orange leukocyte fluorography.

RESULTS. Neutrophil CD11a, CD11b, and CD18 surface integrin levels were 62% (n = 5, P = 0.006), 54% (n = 5, P = 0.045), and 38% (n = 5, P = 0.009) greater in diabetic versus nondiabetic animals, respectively. Seventy-five percent more neutrophils from diabetic versus nondiabetic animals adhered to rat endothelial cell monolayers (n = 6, P = 0.02). Pretreatment of leukocytes with either anti-CD11b or anti-CD18 antibodies lowered the proportion of adherent diabetic neutrophils by 41% (n = 6, P = 0.01 for each treatment), whereas anti-CD11a antibodies had no significant effect (n = 6, P = 0.5). In vivo, systemic administration of anti-CD18 F(ab)’2 fragments decreased diabetic retinal leukostasis by 62% (n = 5, P = 0.001).

CONCLUSIONS. Neutrophils from diabetic animals exhibit higher levels of surface integrin expression and integrin-mediated adhesion. In vivo, CD18 blockade significantly decreases leukostasis in the diabetic retinal microvasculature. Integrin adhesion molecules may serve as therapeutic targets for the treatment and/or prevention of early diabetic retinopathy. (Invest Ophthalmol Vis Sci. 2000;41:1153–1158)

Leukocyte–endothelial cell interactions in tissues are mediated by adhesion molecules expressed on the surface of leukocytes and endothelial cells.1,2 Immunoglobulin superfamily molecules such as intercellular adhesion molecule-1 (ICAM-1) are expressed on endothelial cells and bind to β₂-integrins expressed on leukocytes.1,3 The integrins are transmembrane receptors that consist of noncovalently bound heterodimers composed of α- and β-chains.4,5 The β₂-integrins are operative in leukocyte adhesion and include LFA-1 (lymphocyte function–associated antigen, CD11a/CD18), Mac-1 (leukocyte adhesion receptor, CD11b/CD18), and p150/95 (CD11c/CD18). Each of the β₂-integrins has a common β-chain in combination with a unique α-chain. Lawrence et al.6 have previously shown that CD18 is required for the firm attachment of healthy human neutrophils to human umbilical vein endothelial cells.

There is growing evidence that leukocyte adhesion plays an important role in the pathogenesis of diabetic retinopathy. In a rat model of experimental diabetic retinopathy, Schroeder et al.7 demonstrated retinal capillary occlusion by neutrophils and monocytes in histologic sections. Adjacent to the static leukocytes were areas of endothelial cell damage, capillary loss, and leukocyte extravasation. In a postmortem study of human subjects, Lutty and coworkers8 demonstrated increased numbers of neutrophils in the choroid and retina of diabetic individuals. Expression of ICAM-1, the best characterized endothelial receptor for leukocyte β₂-integrins (CD11/CD18),9

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was elevated in the diabetic retinal vasculature as assessed by immunohistochemistry, a result consistent with an adhesive mechanism mediating the retinal leukocytosis. In a separate study, Hatchell and coworkers\textsuperscript{10} showed that leukocytes from diabetic cats are less deformable than those from nondiabetic cats. Because leukocytes must deform to traverse the smallest retinal capillaries,\textsuperscript{7} stiff leukocytes may contribute to the development of capillary nonperfusion.

In vivo studies from our laboratory have further investigated the role of leukocytes in diabetic retinopathy. Using acridine orange leukocyte fluorography, the density of static leukocytes in the retinas of streptozotocin-induced diabetic rats was demonstrated to be increased.\textsuperscript{11,12} Retinal leukocyte stasis (leukostasis) was observed within 3 days of diabetes induction and was temporally and spatially correlated with capillary nonperfusion and blood-retinal barrier breakdown. The onset of retinal leukostasis coincided with the upregulation of retinal ICAM-1 expression. Causality was demonstrated when an anti-ICAM-1 antibody prevented the diabetes-associated increases in retinal leukostasis and vascular leakage by 48.5% and 85.6%, respectively.\textsuperscript{12} However, the identities and bioactivities of the neutrophil adhesion molecules mediating diabetic retinal leukostasis are less well understood.

The aim of the present study was to investigate in greater detail the role of neutrophils in early diabetic retinal leukostasis. A time point of 1 week of diabetes was chosen in this study because steady state increases in diabetic retinal leukostasis and ICAM-1 expression are achieved in 1 week.\textsuperscript{12} Because adhesion can occur in the absence of increased adhesion molecule expression, both adhesion molecule expression and bioactivity were examined. Finally, the role of CD18 in the development of diabetic retinal leukostasis was examined in vivo using acridine orange leukocyte fluorography and neutralizing anti-CD18 F(ab')\textsubscript{2} fragments.

**METHODS**

**Animals**

Male Long-Evans rats weighing approximately 200 g were used for these experiments. All protocols followed the ARVO Statement on the Use of Animals in Ophthalmic and Vision Research and were approved by the Animal Care and Use Committees of Children's Hospital and Joslin Diabetes Center. The rats were fed standard laboratory chow and allowed free access to water in an air-conditioned room with a 12-hour-12-hour, light-dark cycle.

**Induction of Diabetes**

Rats received a single 60 mg/kg intraperitoneal injection of streptozotocin (Sigma, St. Louis, MO) in 10 mM sodium citrate buffer, pH 4.5, after an overnight fast. Control nondiabetic animals received citrate buffer alone. Animals with blood glucose levels greater than 250 mg/dl 24 hours after injection were considered diabetic. All experiments were performed 1 week after the induction of diabetes.

**Monoclonal Antibodies and F(ab')\textsubscript{2} Fragments**

The monoclonal antibodies (mAbs) were murine in origin and were used as purified IgG. For the in vitro studies, mAbs WT.1 (anti-rat CD11a), 6G2 (anti-rat CD18), and MRC OX-42 (anti-rat CD11b) were obtained from Serotec Inc. (Raleigh, NC). FITC-conjugated mouse IgG\textsubscript{1} mAb isotype control was obtained from PharMingen (San Diego, CA). Fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse IgG\textsubscript{1} Ab was obtained from Caltag Laboratories (Burlingame, CA). For the in vivo studies, WT.3 anti-rat LFA-1 beta chain (CD18) F(ab')\textsubscript{2} fragments were obtained from Behring (Division of Associate of Cape Cod, Inc., Falmouth, MA). Purified mouse anti-human IgG F(ab')\textsubscript{2} fragments were obtained from Jackson Immunoresearch Laboratories (West Grove, PA).

**Flow Cytometry**

The surface expression of CD11a, CD11b, and CD18 on rat neutrophils was determined using flow cytometry as previously described.\textsuperscript{13} Briefly, whole blood anticoagulated with EDTA was obtained from the hearts of rats anesthetized with inhaled isofluorane. Leukocytes were isolated by dextran sedimentation and hypotonic lysis of contaminating erythrocytes. Aliquots of 5 x 10\textsuperscript{5} cells in 100 \mu l RPMI 1640 medium (Bio-Whittaker, Walkersville, MD) containing 5% fetal bovine serum (FBS; RPMI-5%) were incubated on ice for 10 minutes. The tubes were centrifuged at 400g for 5 minutes at 4°C. The cell pellets were resuspended in 100 \mu l RPMI-5% containing 20 \mu g/ml primary mAb to CD11a, CD11b, CD18 or isotype control and incubated for 45 minutes on ice. Primary mAb were detected with FITC-conjugated goat anti-mouse IgG\textsubscript{1} Ab as previously detailed.\textsuperscript{14} The fluorescence of 10\textsuperscript{6} cells was measured on a FACScan (Becton Dickinson, San Jose, CA). Neutrophils were manually gated on the basis of their characteristic forward and side light-scattering properties. The surface expression is presented as the mean channel fluorescence on a logarithmic scale.

**Endothelial Cell–Neutrophil Adhesion Assays**

Peripheral blood was obtained from rats anesthetized with inhaled isofluorane via heart puncture with a 16-gauge EDTA-flushed needle. Neutrophils were isolated from whole blood by density gradient centrifugation with NIM·2 (Neutrophil Isolation Media; Cardinal Associates, Santa Fe, NM) according to the manufacturer’s instructions. Preparations contained >94% neutrophils as determined by eosin and methylene blue staining (Leukostat staining system; Fischer Scientific, Pittsburgh, PA). There was no red blood cell contamination. The cells were used immediately after collection.

The adhesion of unstimulated neutrophils to confluent monolayers of rat prostate endothelial cells (RPECs) was determined under static conditions as previously described.\textsuperscript{14,15} RPECs were obtained from the American Type Culture Collection (ATCC; Manassas, VA) and cultured in Eagle’s minimum essential media (ATCC) supplemented with 5% FBS (GIBCO, Gaithersburg, MD) and 0.3 ng/ml porcine intestinal heparin (Sigma). RPECs were grown to confluence on tissue culture-treated plastic microtiter 96-well plates, stimulated for 24 hours with 30 ng/ml recombinant human tumor necrosis factor-alpha (TNF-\alpha; Genzyme, Cambridge, MA), and incubated for 15 minutes with RPMI-5%. TNF-\alpha stimulation of ICAM-1 surface expression was used for all experiments. Neutrophils were resuspended at 2 x 10\textsuperscript{6} cells/ml in RPMI-5% and incubated for 10 minutes at 37°C with 1 \mu M of the fluorescent marker, 2',7'-bis-(2-carboxyethyl)-5 (and 6) carboxyfluorescein, acetoxymethyl ester (Molecular Probes, Eugene, OR) in dimethyl sulfoxide (vehicle). Fluorescent labeled neutrophils
were washed once and then incubated in RPMI–5% alone or RPMI–5% with a saturating concentration of mAbs (30 μg/ml) to CD11a, CD11b, or CD18 for 10 minutes at room temperature. The neutrophils were washed and then incubated (2 × 10⁶ neutrophils/ml, 50 μl per well) with RPEC for 10 minutes at 37°C. Nonadherent cells were removed, and the content of the wells was lysed with 10 mM Tris-HCl, pH 8.4, containing 0.1% SDS. Fluorescence was determined in a microtiter plate fluorometer (excitation 485 nm, emission 530–540 nm), and the adhesion was reported as the number of adherent neutrophils/mm².

Acridine Orange Leukocyte Fluorography

Leukocyte dynamics in the retina were studied with acridine orange leukocyte fluorography (AOLF).11,12,16,17 Rats were anesthetized with 4 mg/kg xylazine hydrochloride (Phoenix Pharmaceutical, St. Joseph, MO) and 25 mg/kg ketamine hydrochloride (Parke-Davis, Morris Plains, NJ). The day before leukocyte dynamics were observed, a heparin-lock catheter was surgically implanted in the right jugular vein of each rat. The catheter was subcutaneously externalized to the back of the neck. Rats received intravenous injections of 5 mg/kg anti-rat beta chain (CD18, WT.3) F(ab)² fragments or 5 mg/kg anti-human IgG isotype control F(ab)² fragments in sterile phosphate-buffered saline 24 hours before AOLF was performed. The experiments were carried out in a masked fashion.

Immediately before AOLF, each rat was again anesthetized, and the pupil of the left eye was dilated with 1% tropicamide (Alcon, Humancao, Puerto Rico) to observe leukocyte dynamics. A focused image of the peripapillary fundus of the left eye was obtained with a scanning laser ophthalmoscope (Rodenstock Instruments, Munich, Germany). Acridine orange (Sigma) was dissolved in sterile saline (1.0 mg/ml), and 3 mg/kg was injected through the jugular vein catheter at a rate of 1 ml/min. The fundus was observed with the scanning laser ophthalmoscope using the argon blue laser as the illumination source and the standard fluorescein angiography filter in the 40° field setting for 1 minute. Twenty minutes later, the fundus was again observed to evaluate leukostasis in the retina. The images were recorded on videotape at the rate of 30 frames/sec. The video recordings were analyzed on a computer equipped with a video digitizer (Radius, San Jose, CA) that digitizes the video image in real time (30 frames/sec) to 640 × 480 pixels with an intensity resolution of 256 steps. For evaluating retinal leukostasis, an observation area around the optic disc measuring five disc diameters in radius was determined by drawing a polygon bordered by the adjacent major retinal vessels. The density of trapped leukocytes was calculated by dividing the number of static leukocytes (recognized as fluorescent dots) by the area of the observation region (in pixels). The density of static leukocytes was calculated in 8 to 10 peripapillary observation areas and an average density (×10⁻⁵ cells/pixel²) was obtained.

Blood pressures and heart rates were measured using a noninvasive cuff sensor and monitoring system (Ueda Electronics, Tokyo, Japan). Blood anticoagulated with EDTA was drawn from the abdominal aorta of each rat after the experiment to determine the leukocyte count using a hematology analyzer.

Statistical Analysis

All results are expressed as means ± SD. The data were compared by analysis of variance with post hoc comparisons tested using Fisher’s protected least significant difference procedure. Differences were considered statistically significant when P values were <0.05.

RESULTS

Increased Surface Integrin Expression on Diabetic Neutrophils

Integrin expression was measured on the surface of neutrophils from normal and diabetic rats. As shown in Table 1, the flow cytometric analyses demonstrated statistically significant increases in the diabetic leukocyte CD11a, CD11b, and CD18 levels, as evidenced by the increases in mean channel fluorescence. Neutrophil CD11a, CD11b, and CD18 levels were 62% (n = 5, P = 0.006), 54% (n = 5, P = 0.045), and 38% (n = 5, P = 0.009) greater, respectively, on the 1-week diabetic leukocytes versus the nondiabetic leukocytes. Integrin expression was similarly increased on 2-week diabetic neutrophils with CD11a, CD11b, and CD18 levels being 53%, 24%, and 38% greater, respectively (data not shown).

Increased Adhesion of Diabetic Neutrophils to TNFα-Activated Endothelial Cell Monolayers

The functional adhesion of purified neutrophils to cultured endothelial cell monolayers was investigated. Adhesion assays were performed by adding diabetic or nondiabetic neutrophils to TNFα–stimulated rat endothelial cell monolayers under static conditions. TNFα was added to maximize endothelial cell ICAM-1 expression. Preliminary experiments demonstrated a 2.7-fold increase in endothelial cell ICAM-1 expression with TNFα (n = 4, P < 0.0001; data not shown). As shown in Figure 1, 75% more neutrophils from the diabetic rats adhered to the endothelial cell monolayers than neutrophils isolated from nondiabetic rats (n = 6, P = 0.02).

The β₂-integrin molecules mediating neutrophil adhesion in vitro were examined. Neutrophils were pretreated with a panel of anti-CD18 (β₂-integrin) mAbs. In a representative experiment shown in Figure 2, untreated diabetic neutrophils exhibited increased adhesion to TNFα-activated endothelial cell monolayers. Pretreatment with anti-CD11b or anti-CD18 antibodies each decreased diabetic neutrophil adhesion by 41% (n = 6, P = 0.01 for each treatment). In contrast, pretreatment with the anti-CD11a antibody did not significantly affect diabetic neutrophil adhesion (n = 6, P = 0.5 vs. untreated diabetic neutrophils). Moreover, treatment with an equimolar mixture of anti-CD11a, anti-CD11b, and anti-CD18 monoclonal antibodies reduced diabetic neutrophil adhesion by 72% (n =

TABLE 1. Flow-Cytometric Analysis of Integrin Molecule Expression on Neutrophils

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Diabetes</th>
<th>P Value</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD11a</td>
<td>115.0 ± 12.8</td>
<td>185.9 ± 18.5</td>
<td>0.006</td>
<td>5</td>
</tr>
<tr>
<td>CD11b</td>
<td>182.6 ± 39.2</td>
<td>281.9 ± 84.9</td>
<td>0.045</td>
<td>5</td>
</tr>
<tr>
<td>CD18</td>
<td>193.2 ± 34.2</td>
<td>267.1 ± 34.3</td>
<td>0.009</td>
<td>5</td>
</tr>
</tbody>
</table>

Values are means ± SD of mean channel fluorescence.
6, \( P < 0.0001 \) vs. untreated diabetic neutrophils). Nondiabetic neutrophil adhesion also was reduced with the anti-CD11a, anti-CD11b, and anti-CD18 antibodies, as well as with the anti-CD11a/CD11b/CD18 antibody cocktail. The decreases were 39\%, 49\%, 53\%, and 52\%, respectively (\( n = 6, P < 0.05 \) for each treatment vs. untreated nondiabetic neutrophils).

**Decreased Leukostasis in Diabetic Rat Retinas by In Vivo CD18 Blockade**

Retinal leukostasis in living animals was measured with AOLF. Intravenous injection of acridine orange causes leukocytes and endothelial cells to fluoresce through the noncovalent binding of the molecule to double-stranded DNA.\(^{11,16,17}\) When a scanning laser ophthalmoscope is used, retinal leukocytes within blood vessels can be visualized in vivo. Twenty minutes after acridine orange injection, static leukocytes in the capillary bed can be observed as fluorescent dots.\(^{11}\) These labeled cells are leukocytes because blocking CD18, expressed on leukocytes but not on endothelial cells, causes them to disappear (see below).

Leukocyte dynamics in the retina were observed after CD18 F(ab')\(_2\) blockade as shown in the representative photographs of Figure 3. As expected, retinal leukostasis was increased in the diabetic versus nondiabetic rat retinas (Fig. 3B vs. 3A). Treatment of the diabetic rats with the isotype control F(ab')\(_2\) fragments did not lead to detectable changes in the degree of leukostasis (Fig. 3C vs. 3B). However, treatment with the anti-CD18 F(ab')\(_2\) fragments led to a striking decrease in retinal leukostasis (Fig. 3D vs. 3C). Measurements of leukostasis were obtained throughout the entire retinas to avoid any potential sampling error and the means and SDs from independent experiments were compared (Fig. 4). This confirmed that anti-CD18 blockade significantly decreased leukostasis in diabetic rats by 62\% (\( n = 5, P = 0.001 \) vs. animals receiving control F(ab')\(_2\)) (Fig. 4). The body weight, plasma glucose level, blood pressure, and leukocyte counts for the control and diabetic animals are shown in Table 2. The diabetic animals all had significantly elevated blood glucose levels and decreased body weight compared with the normal rats, as is the norm. Blood pressure was similar among groups. The peripheral leukocyte counts in the diabetic anti-CD18, F(ab')\(_2\)-treated animals were increased compared to the untreated diabetic animals, a result consistent with successful CD18 blockade.

**DISCUSSION**

In this study, CD11a, CD11b, and CD18 \( \beta_2 \)-integrin levels were increased on the surface of neutrophils from diabetic rats. The increases correlated with the enhanced functional adhesiveness of diabetic neutrophils to rat endothelial cell monolayers. Similarly, in an in vivo model of experimentally induced diabetes, use of anti-CD18 F(ab')\(_2\) fragments significantly decreased diabetic retinal leukostasis by 62\%, confirming the relevance of these in vitro findings. Taken together, the data suggest that the Mac-1–integrin complex is operative in the adhesion of diabetic neutrophils to the retinal capillary endo-

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**Figure 1.** Adhesion of control and diabetic rat neutrophils to confluent TNF-activated rat endothelial cell monolayers under static conditions. Neutrophils isolated from diabetic rats demonstrated significantly increased adhesion to rat endothelial cell monolayers. All data shown are means ± SD.

**Figure 2.** The effect of anti-integrin antibodies on neutrophil adhesion in vitro. Neutrophils were preincubated with anti-CD11a, anti-CD11b, anti-CD18 (30 \( \mu \)g/ml of each mAb), or an equimolar mixture of anti-CD11a/CD11b/CD18 antibodies before their use in the adhesion studies. Diabetic neutrophils demonstrated increased adhesion to TNF-α-activated endothelial cell monolayers under all treatment conditions. Diabetic neutrophil adhesion was significantly reduced by pretreatment with anti-CD11b, anti-CD18, or the mixture anti-CD11a/CD11b/CD18 antibodies (\( P < 0.05 \) for each condition vs. untreated diabetic neutrophils). Pretreatment with anti-CD11a did not reduce the adhesion of diabetic neutrophils (\( P = 0.5 \)). Control nondiabetic neutrophil adhesion was also significantly reduced with all antibody treatments (\( P < 0.05 \)). All data shown are means ± SD.
helium. Because a major ligand for Mac-1 is ICAM-1, these results are consistent with our previous work showing that ICAM-1 blockade prevents diabetic retinal leukostasis and blood–retinal barrier breakdown.

The results of the in vitro blocking adhesion studies suggest that Mac-1 is the predominant CD18 integrin involved in diabetic neutrophil adhesion to activated RPEC monolayers. At present, the reason for a lack of a CD11a-dependent component in diabetic versus nondiabetic neutrophil adhesion is not known. We speculate that the residual non–CD18-dependent neutrophil adhesion may be due to the VLA4–VCAM adhesion pathway because rat neutrophils constitutively express VLA4 on their surface.

These data strongly support the concept that the leukocyte adhesive changes in this model of diabetes are of a systemic nature. The assayed neutrophils were isolated from the peripheral blood and therefore reflected systemic neutrophil adhesion molecule expression and bioactivity. The causes of the surface integrin changes remain unknown; however, they are likely to be linked to hyperglycemia. For example, hyperglycemia has a direct impact on TNFα expression, a cytokine known to activate integrin adhesion molecules on leukocytes. In vitro work also has shown that hyperglycemia promotes increased leukocyte adhesion to endothelium via ICAM-1 and CD18. Thus, hyperglycemia, either directly or indirectly, is likely a proximal stimulus for the ICAM-1 and CD18 upregulation seen in diabetes.

Another observation in these studies is that a low-level retinal leukostasis occurs in the normal state. The same molecules that are operative in the diabetic state also mediate this presumably normal phenomenon. If the low-level leukostasis in the nondiabetic state is physiologic, then the specificity of an anti-integrin therapy may be compromised.

Finally, these results provide additional evidence of leukocyte involvement in the pathogenesis of diabetic retinopathy. The aggregate data suggest that diabetic retinopathy should be, in one sense, redefined as an inflammatory disease. Very early in diabetes, leukocytes adhere to the vascular endothelium, trigger breakdown of the blood–retinal barrier, impede flow, and in some instances, extravasate into the retinal parenchyma.

In summary, the identification of Mac-1 as a functional adhesive molecule in diabetic retinopathy may provide a po-
tential target for the prevention and/or treatment of the disease. The effect of ICAM-1 and Mac-1 inhibition on the development of diabetic retinopathy deserves further investigation.

References