

# Role of $\alpha 4$ Integrin (CD49d) in the Pathogenesis of Diabetic Retinopathy

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**PURPOSE.** The pathophysiology of diabetic retinopathy is mediated by leukocyte adhesion to the vascular endothelium of the diabetic retina, which results in endothelial injury, blood-retina barrier breakdown, and capillary nonperfusion. Leukocyte adhesion is triggered by the interaction of vascular endothelium adhesion molecules, such as ICAM-1, with leukocyte integrins, such as CD18. Inhibition of ICAM-1/CD18 signaling suppresses but does not completely abolish the cardinal manifestations of diabetic retinopathy, suggesting a role for additional adhesion molecules. Integrin  $\alpha 4$  (CD49d), in complex with integrin  $\beta 1$ , forms very late antigen-4 (VLA-4), which interacts with vascular cell adhesion molecule-1. The authors have now studied the role of integrin  $\alpha 4$ /CD49d in the pathogenesis of diabetic retinopathy.

**METHODS.** Diabetes mellitus was induced in Long Evans rats with streptozotocin, and an anti- $\alpha 4$  integrin/CD49d neutralizing antibody was injected 5 and 10 days later. Two weeks after streptozotocin administration, vascular leakage was quantified with the Evans Blue technique. Leukostasis was measured with a static adhesion assay *ex vivo* and the FITC-lectin perfusion method *in vivo*. Retinal VEGF and TNF- $\alpha$  levels and NF- $\kappa$ B activity were measured by ELISA.

**RESULTS.** Blockade of  $\alpha 4$  integrin/CD49d attenuated the diabetes-induced upregulation of NF- $\kappa$ B activation, VEGF, and TNF- $\alpha$  protein levels and reduced significantly diabetes-induced leukocyte adhesion and vascular leakage.

**CONCLUSIONS.** These data identify  $\alpha 4$  integrin/CD49d as a mediator of leukocyte adhesion and the resultant early signature abnormalities of diabetic retinopathy. Inhibition of this signaling pathway may hold promise for clinical activity in patients with diabetes. (*Invest Ophthalmol Vis Sci.* 2009; 50:4898–4904) DOI:10.1167/iovs.08-2013

Animal models of diabetic retinopathy (DR) successfully recapitulate the pathology of human DR, which is considered to be a chronic inflammatory disease.<sup>1</sup> The inflammatory cascade is initiated with the cytokine-induced activation of

vascular endothelial cells, which triggers the expression of adhesion molecules leading to increased leukocyte migration through the cell junctions into the abluminal surface of the vessels.<sup>2</sup> Leukocyte adhesion contributes to endothelial cell dysfunction and death and results in early blood-retina barrier breakdown, capillary nonperfusion, and retinal ischemia, marking the onset of proliferative DR.<sup>3</sup>

During the inflammatory process, leukocytes interact with the extracellular matrix and the vascular endothelium through an integrin family of membrane receptors that are mainly heterodimers composed of two subunits, an  $\alpha$  and a  $\beta$  subunit.<sup>4</sup> These interactions are transient and dynamic and allow the leukocytes to search for antigens, respond to inflammatory stimuli, extravasate, or adhere firmly to the endothelium to perform immune functions.<sup>5</sup> The initial steps of leukocyte extravasation, such as rolling and tethering, are controlled by adhesion molecules such as P-selectin, E-selectin, and L-selectin.<sup>6</sup> A more firm adhesion is then mediated by the interaction of the adhesion molecules intracellular adhesion molecule (ICAM)-1 and vascular adhesion molecule (VCAM)-1 in the vascular endothelium with CD11a-c/CD18 and  $\alpha 4\beta 1$  integrin (very late antigen [VLA])-4 on the leukocyte surface, respectively.<sup>7</sup> Evidence indicates that the VLA-4/VCAM-1 interaction may also participate in the tethering process and in the firm adhesion of leukocytes to endothelium.<sup>8</sup>

The  $\alpha 4$  integrins ( $\alpha 4\beta 1$  and  $\alpha 4\beta 7$ ) are heterodimer adhesion molecules that are expressed primarily in hematopoietic cells and that mediate the interactions between cells and the extracellular matrix.<sup>5</sup> To allow for dynamic and rapid interactions, the integrin receptors undergo conformational changes and rapid transitions between different states of activation. These changes can be triggered by the engagement of other cell surface receptors or divalent cations (inside-out signaling) or from environmental stimuli (outside-in signaling).<sup>9,10</sup> Several chemokines and cytokines can rapidly and transiently increase  $\alpha 4$  integrin-mediated cell adhesion, including stromal cell-derived factor-1 $\alpha$ <sup>11</sup> and transforming growth factor- $\beta$  (TGF- $\beta$ ).<sup>12</sup>

VLA-4 consists of two chains,  $\alpha 4$  and  $\beta 1$ , that are linked together to form a heterodimer. VLA-4 provides costimulatory signals for T-cell activation, differentiation, and interaction with the antigen-presenting cells.<sup>13</sup> It is believed that different stimuli can regulate VLA-4 activity in similar or distinct fashion, leading to potentially different postadhesion events, such as cell migration or intracellular signaling.<sup>14</sup> VCAM-1 is an endothelial adhesion molecule that is expressed in the activated endothelium, but it is controversial whether it is also expressed in macrophages, follicular cells, and bone marrow dendritic cells. Although the signal transduction pathway that is activated after the binding of VLA-4 to its endothelial counterreceptor, VCAM-1, is not fully elucidated, there is evidence to support the involvement of members of the Ras superfamily, such as Vav1 and Rac.<sup>6</sup> It has been shown that the phosphatidylinositol 3-kinase (PI3K)/Akt kinase axis is activated,<sup>12</sup> whereas the role of p38 and p42/44 MAPK is controversial.<sup>15</sup> There is also evidence of downstream activation of the transcription factors AP-1 and NF- $\kappa$ B upon the VLA-4/VCAM-1 binding that could increase the levels of factors such as VEGF,

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providing a positive feedback for further activation of the VLA-4/VCAM-1 adhesion cascade.<sup>16,17</sup>

ICAM-1 mRNA and protein levels are increased early in the course of DR and contribute significantly to the adhesion of leukocytes to the diabetic vascular endothelium, and, subsequently, endothelial cell apoptosis.<sup>3</sup> Administration of a neutralizing antibody against ICAM-1 suppressed leukocyte adhesion, endothelial cell death, and vascular leakage in our murine model of DR but did not abolish it completely.<sup>3</sup> These data suggested that another set of adhesion molecules are also operative in DR. VLA-4 has been implicated in the pathogenesis of several autoimmune and inflammatory processes, including insulinitis,<sup>18</sup> endotoxin-induced uveitis,<sup>19</sup> nephritis, allergic conjunctivitis, encephalomyelitis, and Crohn's disease,<sup>7</sup> whereas VCAM-1 levels are increased in proliferative DR in humans.<sup>20–22</sup> We now studied the role of  $\alpha 4$  integrin/CD49d in the pathogenesis of DR.

## MATERIALS AND METHODS

### Animals

Male Long-Evans rats weighing approximately 200 g each were used in all experiments. All protocols abided by the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and were approved by the Animal Care and Use Committee of the Massachusetts Eye and Ear Infirmary. The animals were fed standard laboratory chow and allowed free access to water in an air-conditioned room with a 12-hour light/12-hour dark cycle. Unless otherwise stated, the animals were anesthetized with ketamine (40 mg/kg; Ketalar, Parke-Davis, Morris Plains, NJ) and xylazine (4 mg/kg; Rompun, Bayer Leverkusen, Germany) before all experiments.

### Rat Model of Streptozotocin-Induced Diabetes

After a 12-hour fast, animals received a single 60 mg/kg intraperitoneal injection of streptozotocin (STZ; Sigma, St. Louis, MO) in 10 mM sodium citrate buffer, pH 4.5, or citrate buffer alone. Twenty-four hours later, animals with blood glucose levels greater than 250 mg/dL were considered diabetic. Blood glucose and weight measurements were performed on days 0, 1, 6, 10, and 14. In vivo experiments were performed 2 weeks after the induction of diabetes, and blood glucose was measured again before the onset of the experiments to confirm the diabetic or nondiabetic state. All in vivo experiments were performed in a masked fashion. In previous studies, our laboratory had demonstrated that STZ non-converters do not differ in terms of gene expression, amount of blood-retinal barrier breakdown, or leukostasis from nondiabetic controls. Thus, in the present study, nondiabetic animals with confirmed glucose levels of less than 120 mg/dL were used as controls.

### Treatment of Diabetic Animals with a Neutralizing Antibody against $\alpha 4$ Integrin/CD49d

Animals with confirmed diabetes were randomly assigned to receive intraperitoneal injections of 1 or 5 mg/kg of a mouse anti-rat CD49d neutralizing antibody (clone TA-2, mouse IgG1; Seikagaku America, Associates of Cape Cod, Inc., East Falmouth, MA) or 1 or 5 mg/kg of an isotype-matched control mouse IgG (clone 15H6; Seikagaku America) on days 5 and 10 after the onset of diabetes. Leukocyte adhesion and vascular permeability were measured 2 weeks after the onset of diabetes. These preliminary experiments showed that the 1 mg/kg dose was in general as effective as the 5 mg/kg dose and was therefore chosen for use in all subsequent experiments. All in vivo experiments were performed in a masked fashion.

### Isolation of Peripheral Blood Mononuclear Cells

Peripheral blood was obtained from diabetic and control rats anesthetized with 50 mg/kg pentobarbital by heart puncture with a 16-gauge

EDTA flashed needle. Peripheral blood mononuclear cells (PBMCs) were isolated from whole blood by density gradient centrifugation with reagent (Histopaque 1083; Sigma, St. Louis, MO) according to the manufacturer's instructions. The preparations contained greater than 90% PBMCs, as determined by eosin and methylene blue staining.

### Ex Vivo Endothelial Cell PBMC Adhesion Assay

The adhesion of unstimulated PBMCs to confluent monolayers of rat prostate endothelial cells (RPECs; American Type Culture Collection, Manassas, VA) was determined under static conditions. In detail, RPECs were cultured in Eagle's minimum essential media (ATCC) supplemented with 5% FBS (Gibco, Gaithersburg, MD) and 0.3 ng/mL porcine intestinal heparin (Sigma). The RPECs were grown to confluence on tissue culture-treated plastic microtiter 96-well plates, stimulated for 24 hours with 30 ng/mL recombinant human TNF- $\alpha$  (Genzyme, Cambridge, MA) to upregulate the expression of adhesion molecules, and incubated for 15 minutes with RPMI 5% FBS. PBMCs were resuspended at  $2 \times 10^6$  cells/mL in RPMI 5% FBS 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). The PBMCs were washed and then incubated ( $2 \times 10^6$  PBMCs/mL, 100  $\mu$ L/well) with a saturating concentration of antibody (30 ng/mL) to  $\alpha 4$  integrin/CD49d or the respective control for 10 minutes at room temperature. The PBMCs were subsequently washed and then incubated ( $2 \times 10^6$  PBMCs/mL, 100  $\mu$ L 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 PBMCs per square millimeter.

### In Vivo Quantification of Retinal Leukostasis

The adhesion of leukocytes in the retinal vasculature in diabetic and control rats that received the anti-CD49d or respective control antibody was assessed with an FITC-based concanavalin A lectin method 2 weeks after the induction of diabetes. After the induction of deep anesthesia in the rat, the chest cavity was opened and a 14-gauge perfusion cannula was introduced to the left ventricle. The right atrium was opened with a 12-gauge needle to achieve outflow. With the heart providing the motive force, 20 mL PBS was administered from the perfusion cannula to remove erythrocytes and nonadherent leukocytes, followed by perfusion with FITC-coupled concanavalin A lectin (20  $\mu$ g/mL in PBS, pH 7.4, 5 mg/kg body weight; Vector Laboratories, Burlingame, CA). The animals were then perfused with 20 mL PBS alone to remove excess concanavalin A. The latter stained adherent leukocytes and the vascular endothelium. The retinas were flat-mounted in a water-based fluorescence anti-fading medium (Fluoromount; Southern Biotechnology, Birmingham, AL) and were imaged by fluorescence microscopy (Axioptan, FITC filter, 40 $\times$ ; Carl Zeiss, Oberkochen, Germany). Leukocyte location was scored as arteriolar, venular, or capillary. The total number of adherent leukocytes per retina was counted. All experiments were performed in a masked fashion.

### Measurement of Retinal Vascular Leakage

The blood-retinal vascular leakage in diabetic and control rats that received the anti-CD49d or the respective control antibody was assessed with the Evans Blue method. After the animals were deeply anesthetized, Evans blue dye (Sigma) dissolved in normal saline (30 mg/mL) was injected through the tail vein over 10 seconds at a dose of 45 mg/kg. After the dye had circulated for 2 hours, the chest cavity was opened, and 1 mL blood was withdrawn from the left ventricle. These blood samples were centrifuged at 12,000 rpm for 15 minutes and diluted to 1/10,000 their initial concentration in formamide (Sigma). Absorbance was measured with a spectrophotometer at 620 nm. The concentration of dye in the plasma was calculated from a standard curve of Evans blue in formamide and represented the Evans Blue

standard concentration. Each rat was subsequently perfused through the left ventricle with citrate buffer (0.05 M, pH 3.5) for 2 minutes at a physiological pressure of 120 mm Hg. The retinas were then carefully dissected under an operating microscope. After measurement of the retinal dry weight, Evans blue was extracted by incubating each retina in 0.3 mL formamide for 18 hours at 70°C. The extract was ultracentrifuged at a speed of 70,000 rpm for 45 minutes at 4°C. Sixty microliters of the supernatant was used for spectrophotometric measurement at 620 nm. Each measurement occurred over a 5-second interval, and all sets of measurements were preceded by evaluation of known standards. Background-subtracted absorbance was determined by measuring each sample at 620 nm (the absorbance peak for Evans blue in formamide) and 740 nm (the absorbance nadir). The concentration of dye in the extracts was calculated from a standard curve of Evans blue in formamide. Blood-retinal barrier breakdown was calculated using the following equation, with results expressed in microliters of plasma per gram of retina (dry weight)  $\times$  hours:

Blood-retinal barrier breakdown

$$= \frac{\text{Evans blue } (\mu\text{g})/\text{retina dry weight (g)}}{\text{Evans blue concentration } (\mu\text{g})/\text{plasma } (\mu\text{L}) \times \text{circulation time (h)}}$$

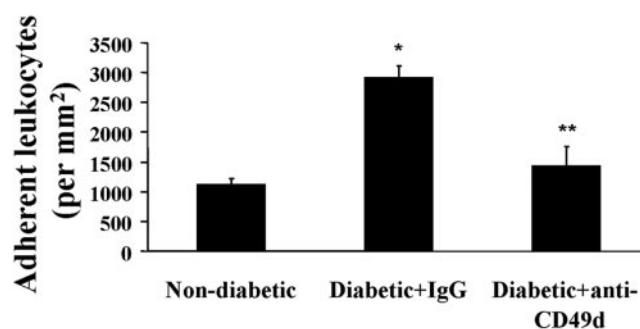
Retinal VEGF and TNF- $\alpha$  levels were measured in control and diabetic rats treated with the anti-CD49d or control antibody 2 weeks after the induction of diabetes with a commercially based ELISA method. In detail, each retina was homogenized in 100  $\mu$ L solution consisting of 20 mM imidazole hydrochloride, 100 mM KCl, 1 mM MgCl<sub>2</sub>, 1 mM EGTA, 1% Triton, 10 mM NaF, 1 mM sodium molybdate, and 1 mM EDTA supplemented with a cocktail of protease inhibitors (Complete; Roche, Basel, Switzerland) before use. Samples were cleared by centrifugation for 10 minutes at 13,000 rpm and assessed for protein concentration with the BCA assay (Mini BCA kit; Pierce Biotechnology, Inc., Rockford, IL). VEGF and TNF- $\alpha$  protein levels were estimated with the respective enzyme-linked immunosorbent assay (ELISA) kits (R&D Systems, Minneapolis, MN) according to the manufacturer's instructions. The reaction was stopped, and absorption was measured in an ELISA reader at 450 nm. All measurements were performed in duplicate. The tissue sample concentration was calculated from a standard curve and corrected for protein concentration.

### Preparation of Nuclear Extracts and NF- $\kappa$ B Activity ELISA

Retinal NF- $\kappa$ B DNA-binding activity levels were measured in control and diabetic rats treated with the anti-CD49d or control antibody 2 weeks after the induction of diabetes. Pooled retinas were homogenized with a mechanical homogenizer in five pellet volumes of buffer A (20 mM Tris, pH 7.6, 10 mM KCl, 0.2 mM EDTA, 20% [by vol] glycerol, 1.5 mM MgCl<sub>2</sub>, 2 mM dithiothreitol [DTT]), 1 mM Na<sub>3</sub>VO<sub>4</sub> and protease inhibitors [Complete TM; Boehringer Mannheim, Mannheim, Germany]). Nuclei were pelleted (2500g, 10 minutes) and resuspended in two pellet volumes of buffer B (identical to buffer A except that KCl was increased to 0.42 M). Nuclei debris was removed by centrifugation (15,000g, 20 minutes), and the supernatant was dialyzed against one change of buffer Z (20 mM Tris-HCl [pH 7.8], 0.1 M KCl, 0.2 mM EDTA, 20% glycerol) for at least 3 hours at 4°C with cassettes (Dialyze Z; Pierce Biotechnology, Inc.). Protein concentration was measured with the BCA assay. Two micrograms of the retinal nuclear extracts were analyzed with a commercially available, ELISA-based assay that measures binding of active NF- $\kappa$ B to DNA (TransAM; NF-B p65 Transcription Factor Assay, Active Motif North America, Carlsbad, CA), according to the instructions of the manufacturer. The peroxidase reaction was quantified at 450 nm with a reference wavelength of 655 nm.

### Statistical Analysis

All results are expressed as mean  $\pm$  SD. Data were compared by ANOVA, with post hoc comparisons tested with Fisher's protected



**FIGURE 1.** PBMCs were isolated from whole blood of control and diabetic animals by density gradient centrifugation, labeled with a fluorescent marker, and incubated with rat prostate endothelial cells on a 96-well plate. After several washings, the adherent PBMCs were derived from the fluorescence of the coculture in a microtiter plate fluorometer (excitation 485 nm, emission 530–540 nm). Anti-CD49d antibody treatment decreased the adhesion of diabetic PBMCs to endothelial cells *ex vivo*. Leukocyte adhesion *ex vivo* increased in leukocytes from diabetic animals ( $2915 \pm 191$  adherent leukocytes from diabetic rats pretreated with the isotype-matched control antibody per square millimeter compared with  $1119 \pm 102$  adherent leukocytes from nondiabetic rats per square millimeter;  $n = 6$ ;  $P < 0.05$ ). Pretreatment with the anti-CD49d antibody reduced leukocyte adhesion ( $1433 \pm 326$  adherent leukocytes from diabetic rats pretreated with the anti-CD49d antibody compared with  $2915 \pm 191$  adherent leukocytes from diabetic rats pretreated with the control antibody per square millimeter;  $n = 6$ ;  $P < 0.05$ ). \* $P < 0.05$  compared with nondiabetic controls. \*\* $P < 0.05$  compared with animals treated with the isotype-matched control antibody.

least significant difference procedure. Differences were considered statistically significant at  $P < 0.05$ .

## RESULTS

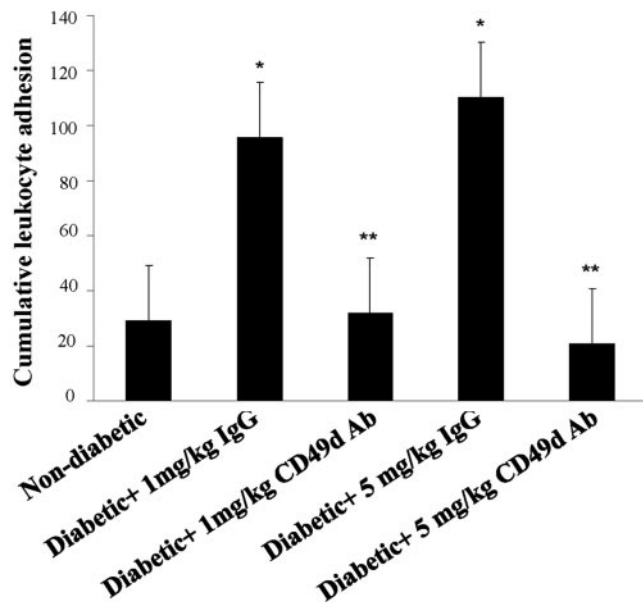
### Diabetes-Induced Upregulation of PBMC Adhesion Ex Vivo to Rat Endothelial Cells Is Blocked by the Administration of CD49d Antibody

Monocytes from subjects with diabetes adhered more efficiently to endothelial cells *ex vivo* than those from subjects without diabetes.<sup>23</sup> We now investigated whether CD49d participates in this phenomenon. In agreement with previous findings, PBMC adhesion *ex vivo* increased in PBMCs from diabetic animals ( $2915 \pm 191$  adherent monocytes from diabetic rats pretreated with the control antibody per square millimeter vs.  $1119 \pm 102$  adherent monocytes from nondiabetic rats per square millimeter;  $n = 6$ ;  $P < 0.05$ ; Fig. 1). Pretreatment with the anti-CD49d antibody reduced PBMC adhesion ( $1433 \pm 326$  adherent monocytes from diabetic rats pretreated with the anti-CD49d antibody vs.  $2915 \pm 191$  adherent PBMCs from diabetic rats pretreated with the control antibody per square millimeter;  $P < 0.05$ ;  $n = 6$ ; Fig. 1).

### Intraperitoneal Administration of CD49d Antibody Decreases Retinal Leukocyte Adhesion In Vivo in the STZ-Induced Diabetic Rat Model

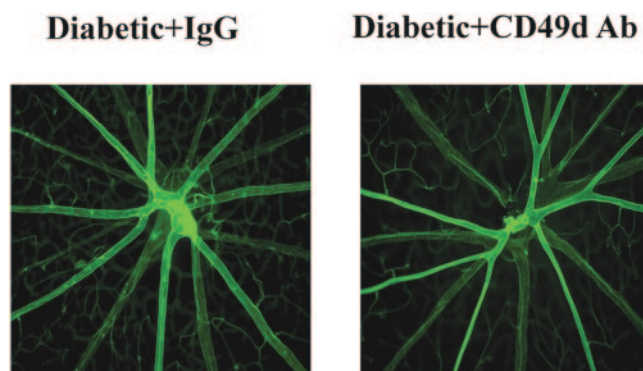
Administration of a neutralizing antibody against ICAM-1 reduces leukocyte adhesion in our STZ-induced rodent diabetes model.<sup>3</sup> In agreement with previously published results, we found that leukocyte adhesion increased from  $29 \pm 4.27$  (total number of adherent leukocytes per retina) in nondiabetic rats ( $n = 9$ ) to  $110.16 \pm 25.84$  in diabetic rats treated with the 5 mg/kg dose of the control antibody ( $P < 0.05$ ,  $n = 18$ ) and  $95.72 \pm 22.79$  in diabetic rats treated with 1 mg/kg dose of the control antibody ( $P < 0.05$ ,  $n = 12$ ; Figs. 2 and 3). Adminis-



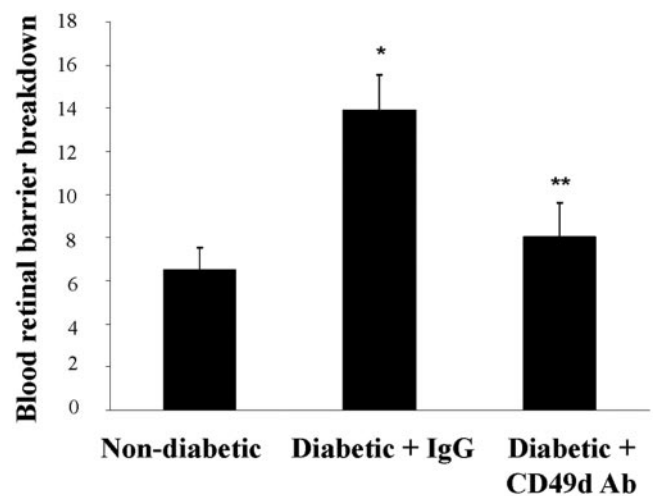


**FIGURE 2.** The adhesion of leukocytes in the retinal vasculature in diabetic and control rats that received the anti-CD49d or the respective control antibody was assessed with an FITC-based concanavalin A lectin method 2 weeks after the induction of diabetes. After the induction of deep anesthesia in each rat and opening of the chest cavity, the right atrium was cannulated and the animal was perfused with PBS and FITC-coupled concanavalin A lectin (20 mg/mL in PBS, pH 7.4). The retinas were flatmounted on a slide and imaged by fluorescent microscopy, and the total amount of adherent leukocytes per retina was counted. Anti-CD49d treatment decreased the adhesion of leukocytes in the retinal vasculature in our STZ-induced rodent diabetes model *in vivo*. Leukocyte adhesion increased from  $29 \pm 4.27$  in nondiabetic rats ( $n = 9$ ) to  $110.16 \pm 25.84$  in rats treated with the 5 mg/kg dose of the control antibody ( $P < 0.05$ ;  $n = 18$ ) and  $95.72 \pm 22.79$  in rats treated with the 1 mg/kg dose of the control antibody ( $P < 0.05$ ;  $n = 12$ ). Administration of the 5 mg/kg dose of the anti-CD49d antibody reduced significantly the number of adherent leukocytes to  $20.5 \pm 11.15$  ( $P < 0.05$ ;  $n = 32$ ), and administration of the 1 mg/kg dose of the anti-CD49d antibody also reduced the number of adherent leukocytes to  $32 \pm 10$  ( $P < 0.01$ ;  $n = 21$ ). \* $P < 0.05$  compared with nondiabetic controls. \*\* $P < 0.05$  compared with animals treated with the isotype-matched control antibody.

tration of the 5 mg/kg dose of the anti-CD49d antibody in diabetic animals reduced significantly the number of adherent leukocytes to  $20.5 \pm 11.15$  ( $P < 0.05$ ,  $n = 32$ ), and adminis-



**FIGURE 3.** Representative pictures from retinal flatmounts prepared from diabetic rats treated with the isotype-matched control IgG (*left*) or the anti-CD49d antibody (*right*) and perfused with FITC-lectin to visualize the adherent leukocytes. Adherent leukocytes were significantly fewer in the diabetic rats treated with the anti-CD49d antibody.



**FIGURE 4.** Blood-retinal vascular leakage in diabetic and control rats that received the anti-CD49d or the respective control antibody was assessed with the Evans blue method. Evans blue dye was injected through the tail vein and allowed to circulate for 2 hours. The animals were subsequently perfused through the left ventricle with citrate buffer and paraformaldehyde under physiological pressure, and the retinas were dissected under an operating microscope. Evans blue was eluted in formamide overnight at 70°C and extracted with centrifugation through filters. The concentration of dye in the extracts was calculated from a standard curve of Evans blue in formamide. Anti-CD49d treatment suppressed the blood-retinal barrier breakdown in our STZ-induced rodent diabetes model. Blood-retinal barrier breakdown increased significantly 2 weeks after the induction of diabetes from  $6.5 \pm 1.06$  in control (nondiabetic) rats to  $13.9 \pm 1.63$  in diabetic rats treated with the control antibody ( $P < 0.05$ ;  $n = 8$ ). Administration of the anti-CD49d neutralizing antibody significantly reduced the diabetes-induced vascular leakage to  $8 \pm 1.6$  in the diabetic animals treated with the anti-CD49d antibody compared with  $13.9 \pm 1.63$  in the diabetic animals treated with the control antibody ( $P < 0.05$ ;  $n = 8$ ). \* $P < 0.05$  compared with the nondiabetic controls. \*\* $P < 0.05$  compared with animals treated with the isotype-matched control antibody.

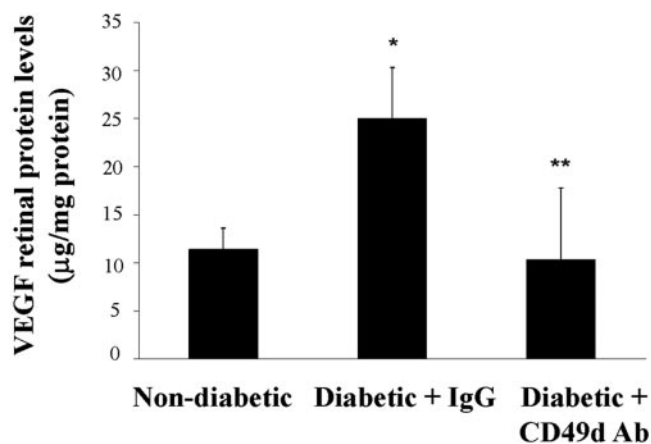
tration of the 1 mg/kg dose of the anti-CD49d antibody also reduced the number of adherent leukocytes to  $32 \pm 10$  ( $P < 0.01$ ,  $n = 21$ ; Figs. 2, 3). Because the 1 mg/kg dose was in general as effective as the 5 mg/kg dose, subsequent experiments were performed with the 1 mg/kg dose.

#### Intraperitoneal Administration of CD49d Antibody Reduces the Blood-Retinal Barrier Breakdown in the STZ-Induced Diabetic Rat Model

Leukocyte adhesion correlates well with the blood-retinal barrier breakdown in the STZ-induced model of rodent diabetes.<sup>1</sup> In agreement with our previously published results, blood-retinal barrier breakdown increased significantly 2 weeks after the induction of diabetes from  $6.5 \pm 1.06$  in control (nondiabetic) rats to  $13.9 \pm 1.63$  in diabetic rats treated with the control antibody ( $P < 0.05$ ,  $n = 8$ ; Fig. 4). Administration of the anti-CD49d neutralizing antibody significantly reduced diabetes-induced vascular leakage to  $8 \pm 1.6$  ( $P < 0.05$ ,  $n = 8$ ; Fig. 4).

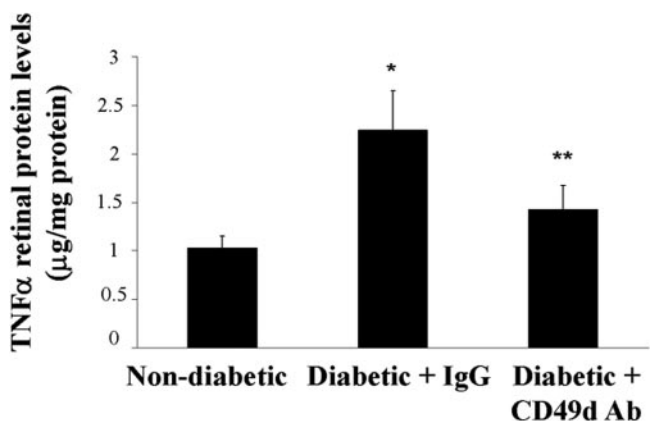
#### Intraperitoneal Administration of CD49d Antibody Reduces the Diabetes-Induced Upregulation of TNF- $\alpha$ and VEGF Protein Levels

VEGF and TNF- $\alpha$  have been implicated in the pathogenesis of DR. Both protein levels increase early in the course of diabetes

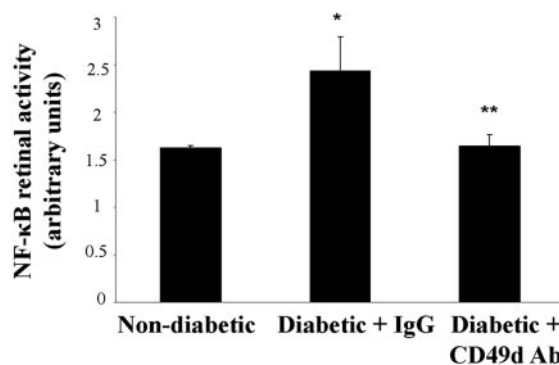


**FIGURE 5.** Retinal VEGF levels were measured in control and diabetic rats treated with the control or the anti-CD49d antibody using a commercially available ELISA method. Anti-CD49d treatment decreased the upregulation of VEGF protein levels in our STZ-induced diabetic rat model. VEGF increased significantly 2 weeks after the induction of diabetes, whereas administration of the anti-CD49d neutralizing antibody reduced significantly the diabetes-induced increase of VEGF protein levels (compared with the isotype-matched IgG antibody control). \* $P < 0.05$  compared with the nondiabetic controls. \*\* $P < 0.05$  compared with animals treated with the isotype-matched control antibody.

in the retina.<sup>24</sup> We next investigated whether VEGF and TNF- $\alpha$  protein levels were affected by the anti-CD49d therapy. In agreement with our previously published results, VEGF and TNF- $\alpha$  levels increased 2 weeks after the onset of diabetes:  $11.4 \pm 2.2$  pg VEGF/mg tissue in the nondiabetic animals compared with  $25 \pm 5.3$  pg VEGF/mg tissue in the diabetic animals and  $1.028 \pm 0.128$  pg TNF- $\alpha$ /mg tissue in the nondiabetic animals compared with  $2.24 \pm 0.41$  pg TNF- $\alpha$ /mg tissue in the diabetic animals ( $P < 0.05$  for both cytokines,  $n = 6$ ; Figs. 5 and 6, respectively). Anti-CD49d treatment reduced significantly the levels of both cytokines to  $10.34 \pm 7.4$  pg



**FIGURE 6.** Retinal TNF- $\alpha$  levels were measured in control and diabetic rats treated with the control or the anti-CD49d antibody, using a commercially available ELISA method. Anti-CD49d treatment decreased the upregulation of TNF- $\alpha$  protein levels in our STZ-induced diabetic rat model. TNF- $\alpha$  increased significantly 2 weeks after the induction of diabetes, whereas administration of the anti-CD49d neutralizing antibody reduced significantly the diabetes-induced induction of TNF- $\alpha$  protein levels (compared with the isotype-matched IgG antibody control). \* $P < 0.05$  compared with the nondiabetic controls. \*\* $P < 0.05$  compared with animals treated with the isotype-matched control antibody.



**FIGURE 7.** Retinal NF- $\kappa$ B activity was measured in control and diabetic rats treated with the control or the anti-CD49d antibody using a commercially available ELISA-based assay. Anti-CD49d treatment decreased the upregulation of NF- $\kappa$ B activation in our STZ-induced diabetic rat model. NF- $\kappa$ B activation increased significantly 2 weeks after the induction of diabetes, whereas administration of the anti-CD49d neutralizing antibody reduced significantly the diabetes-induced increase in NF- $\kappa$ B activation (compared with the isotype-matched IgG antibody control). \* $P < 0.05$  compared with the nondiabetic controls. \*\* $P < 0.05$  compared with animals treated with the isotype-matched control antibody.

VEGF/mg tissue in the diabetic animals treated with anti-CD49d compared with  $25 \pm 5.3$  pg VEGF/mg tissue in the diabetic animals treated with the control antibody and  $1.42 \pm 0.25$  pg TNF- $\alpha$ /mg tissue in the diabetic animals treated with the anti-CD49d antibody compared with  $2.24 \pm 0.41$  pg TNF- $\alpha$ /mg tissue in the diabetic animals treated with the control antibody ( $P < 0.05$  for both cytokines;  $n = 6$ ; Figs. 5 and 6, respectively).

### Intraperitoneal Administration of CD49d Antibody Reduces the Diabetes-Induced Upregulation of NF- $\kappa$ B Activity

Both TNF- $\alpha$  and VEGF gene promoters are under direct stimulatory control from the transcription factor NF- $\kappa$ B. NF- $\kappa$ B activity increases early in the course of diabetes and correlates with both VEGF and TNF- $\alpha$  expression.<sup>24</sup> We next investigated whether CD49d blockade suppresses NF- $\kappa$ B activity in our STZ-induced rodent diabetes model. In agreement with our previously published results, NF- $\kappa$ B activity increased 2 weeks after the onset of diabetes 1.49-fold in the diabetic rats treated with the control antibody compared with the nondiabetic rats ( $P < 0.05$ ,  $n = 6$ ; Fig. 7). Anti-CD49d treatment reduced NF- $\kappa$ B activity to  $1.002 \pm 0.07$  fold induction ( $P < 0.05$  compared with rats treated with the isotype-matched control antibody;  $n = 6$ ; Fig. 7).

### DISCUSSION

In animal models of DR, retinal vascular leakage, vascular damage, and nonperfusion correlate spatially and temporally with increased leukocyte adhesion in the vascular endothelium.<sup>3,25</sup> Our laboratory has previously demonstrated a role for the ICAM-1/CD18 pair of adhesion molecules in the pathogenesis of early DR.<sup>3</sup> However, ICAM-1 inhibition does not completely abolish leukocyte adhesion, emphasizing the importance of other adhesion molecules in DR.<sup>3</sup> We now report that  $\alpha 4$  integrins may play that role. Administration of an anti- $\alpha 4$  integrin antibody attenuated leukocyte adhesion and vascular leakage in our rodent model of DR, suppressed VEGF and TNF- $\alpha$  expression, and reduced NF- $\kappa$ B activity.

VLA-4 ( $\alpha 4\beta 1$  integrin) is expressed on resting lymphocytes, eosinophils, and PBMCs and mediates cell-to-cell adhesion by binding to its ligands VCAM-1 and fibronectin on endothelial cells. Adherent leukocytes in the vascular endothelium can increase vascular permeability by inducing Fas/FasL-mediated apoptotic death of endothelial and supporting cells<sup>26</sup> and by upregulating levels of MCP-1, TNF- $\alpha$ , and VEGF.<sup>27</sup> The reduction of VEGF and TNF- $\alpha$  levels by CD49d blockade can further promote the suppressive effect on the leukocytic adhesion in DR. Given that activated leukocytes are a source of VEGF and TNF- $\alpha$ , this decrease may reflect the impaired transmigration of inflammatory cells through the endothelial barrier into the retina or may result from the observed decreased activation of NF- $\kappa$ B (which regulates their gene expression) and the cause of further NF- $\kappa$ B inhibition.<sup>16,17,28,29</sup> Additionally, the reduction in oxidative stress caused by the suppressed leukocyte activation can further reduce the activation of the redox-sensitive NF- $\kappa$ B and the upregulation of VEGF and TNF- $\alpha$ .<sup>30</sup>

Although the expression of both endothelial adhesion molecules (ICAM-1 and VCAM-1) can be upregulated by the same set of cytokines (including IL-1 and TNF- $\alpha$ ) and they are both involved in leukocyte adhesion, it is not fully understood whether these adhesion molecules exert distinct or overlapping functions in DR. Nevertheless it is conceivable that blockade of both adhesion pathways could have a synergistic beneficial effect on DR.

In this study, we did not directly demonstrate increased activation of the VLA-4 complex, and we could not detect an increase in VLA-4 surface expression in flow cytometry experiments (data not shown). However, our ex vivo and in vivo leukostasis data strongly demonstrate that CD49d-mediated adhesion is significantly increased in leukocytes derived from diabetic animals. This phenomenon may be mediated by changes in VLA-4 conformation and affinity, as described previously in other models. For example, stromal cell-derived factor-1, which is believed to play a significant role in the pathogenesis of DR,<sup>31</sup> can increase VLA-4 affinity for its ligands by inducing a conformational change in the integrin,<sup>32</sup> through PI3K.<sup>33</sup> Alternatively, the phospholipase C pathway, which is activated in diabetes, can promote the high-affinity conformation of  $\alpha 4$  integrins through increased calcium and calmodulin activation.<sup>15,34</sup>

Alternatively, the increased CD49d-mediated leukocyte adhesion in DR may be caused by an upregulation of VLA-4 ligands/counterreceptors. VCAM-1, the classic counterreceptor for VLA-4, was not found to be upregulated in our rodent model of diabetic retinopathy (data not shown). Fibronectin-1, which is upregulated in diabetes, is another counterreceptor for VLA-4 in choroidal vessels that could account for the increased VLA-4 mediated adhesion.<sup>35</sup> Cytokines upregulated during the course of DR, such as TNF- $\alpha$ , change the endothelial cell membrane fluidity, causing an increase in the fibronectin connective segment-1 exposure and therefore an increase in its cellular adhesive properties.<sup>36</sup>

Moreover, the neutralizing antibody that we used is raised against  $\alpha 4$  integrin (CD49d) and recognizes both the  $\alpha 4\beta 1$  complex (VLA-4) and the  $\alpha 4\beta 7$  complex. The mucosal addressin cell adhesion molecule MAdCAM-1, which acts as a counterreceptor for the  $\alpha 4\beta 7$  ligand and plays an important role in T-lymphocyte homing in the intestine,<sup>37</sup> may mediate the increase in CD49d-mediated adhesion in our model. Its role has been established in autoimmune hepatitis and inflammatory bowel disease, and it is expressed in the retina,<sup>38</sup> although its contribution to diabetic retinopathy has not been thoroughly investigated.

In conclusion, we report a role for  $\alpha 4$  integrin/CD49d in the pathogenesis of DR. CD49d blockade suppressed the diabetes-induced upregulation of NF- $\kappa$ B activity and VEGF and TNF- $\alpha$

protein levels, and it decreased vascular leakage and leukostasis. CD49d blockade suppresses inflammation and autoimmune diseases.<sup>7,18,19,39</sup> A humanized anti-VLA-4 mAb (natalizumab) has demonstrated clinical activity in relapsing multiple sclerosis, Crohn's disease, and rheumatoid arthritis,<sup>39</sup> though it has rare but potentially serious side effects when given systemically. Our study points to the effectiveness of an anti-CD49d antibody that may be administered through the intravitreal route in patients with diabetes.

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