Role of α4 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 α4 (CD49d), in complex with integrin β1, forms very late antigen-4 (VLA-4), which interacts with vascular cell adhesion molecule-1. The authors have now studied the role of integrin α4/CD49d in the pathogenesis of diabetic retinopathy.

METHODS. Diabetes mellitus was induced in Long Evans rats with streptozotocin, and an anti-α4 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-α levels and NF-κB activity were measured by ELISA.

RESULTS. Blockade of α4 integrin/CD49d attenuated the diabetes-induced upregulation of NF-κB activation, VEGF, and TNF-α protein levels and reduced significantly diabetes-induced leukocyte adhesion and vascular leakage.

CONCLUSIONS. These data identify α4 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.1 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.2 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.3

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 α and a β subunit.4 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.5 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.6 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 αβ1 integrin (very late antigen [VLA]-4) on the leukocyte surface, respectively.7 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.8 The α4 integrins (α4β1 and α4β7) are heterodimer adhesion molecules that are expressed primarily in hematopoietic cells and that mediate the interactions between cells and the extracellular matrix.9 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).9,10 Several chemokines and cytokines can rapidly and transiently increase α4 integrin-mediated cell adhesion, including stromal cell-derived factor-1α11 and transforming growth factor-β (TGF-β).12

VLA-4 consists of two chains, α and β, 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.13 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 signal transduction.14 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.15 It has been shown that the phosphatidylinositol 3-kinase (PI3K)/Akt kinase axis is activated,12 whereas the role of p38 and p42/44 MAPK is controversial.15 There is also evidence of downstream activation of the transcription factors AP-1 and NF-κB upon the VLA-4/VCAM-1 binding that could increase the levels of factors such as VEGF,
providing a positive feedback for further activation of the VLA-4/VCAM-1 adhesion cascade.\textsuperscript{16,17}

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.\textsuperscript{8} 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.\textsuperscript{5} 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 insulitis,\textsuperscript{18} endotoxin-induced uveitis,\textsuperscript{19} nephritis, allergic conjunctivitis, encephalomyelitis, and Crohn’s disease,\textsuperscript{7} whereas VCAM-1 levels are increased in proliferative DR in humans.\textsuperscript{20–22} We now studied the role of α4 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 α4 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 cosin and methylene blue staining.

**Ex Vivo Endothelial Cell PBMC Adsorption Assay**

The adhesion of unstimulated PBMCs to confluent monolayers of rat proepithelial cell adhesion molecules (REPECs; American Type Culture Collection, Manassas, VA) was determined under static conditions. In detail, REPECs were cultured in Eagle’s minimum essential media (ATCC) supplemented with 5% FBS (Gibco, Gaithersburg, MD) and 0.5 ng/mL porcine intestinal heparin (Sigma). The REPECs were grown to confluence on tissue culture-treated plastic microtiter 96-well plates, stimulated for 24 hours with 30 ng/mL recombinant human TNF-α (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 μM 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 μL/well) with a saturating concentration of antibody (30 ng/mL) to α4 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 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 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 canula 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 canula 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 (Axioplan, FITC filter, 40×; 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 formamidene (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 formamidene 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 0.5 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 7-40 nm (the absorbance nadir). The concentration of dye in the extract 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) × hours:

**Blood-retinal barrier breakdown**

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\text{Evans blue (µg)/retina dry weight (g) = Evans blue concentration (µg)/plasma (µL) \times circulation time (h)}
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Retinal VEGF and TNF-α 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 µL solution consisting of 20 mM imidazole hydrochloride, 100 mM KCl, 1 mM MgCl₂, 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 assayed for protein concentration with the BCA assay (Mini BCA kit; Pierce Biotechnology, Inc., Rockford, IL). VEGF and TNF-α 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-κB Activity ELISA**

Retinal NF-κB 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 B (20 mM Tris, pH 7.6, 10 mM KCl, 0.2 mM EDTA, 20% [by vol] glycerol, 1.5 mM MgCl₂, 2 mM dithiothreitol [DTT]), 1 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 assayed for protein concentration with the BCA assay (Mini BCA kit; Pierce Biotechnology, Inc., Rockford, IL). VEGF and TNF-α 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.

**Statistical Analysis**

All results are expressed as mean ± SD. Data were compared by ANOVA, with post hoc comparisons tested with Fisher’s protected 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 from those subjects without diabetes. 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 ± 191 adherent leukocytes from diabetic rats pretreated with the control antibody per square millimeter compared with 1119 ± 102 adherent leukocytes from nondiabetic rats per square millimeter; n = 6; P < 0.05). Pretreatment with the anti–CD49d antibody reduced leukocyte adhesion (1433 ± 326 adherent leukocytes from diabetic rats pretreated with the anti–CD49d antibody compared with 2915 ± 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.

**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. In agreement with previously published results, we found that leukocyte adhesion increased from 29 ± 4.27 (total number of adherent leukocytes per retina) in nondiabetic rats (n = 9) to 110.16 ± 25.84 in diabetic rats treated with the 5 mg/kg dose of the control antibody (P < 0.05, n = 18) and 95.72 ± 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 1](https://iovs.arvojournals.org/pdfaccess.ashx?url=/data/journals/iovs/933243/)
Anti–CD49d antibody reduced significantly the number of adherent leukocytes to 32 in diabetic animals compared with nondiabetic controls. **P < 0.05 compared with nondiabetic controls. **P < 0.05 compared with animals treated with the isotype-matched control antibody.

Intraperitoneal Administration of CD49d Antibody Reduces the Diabetes-Induced Vascular Leakage in the Rat Model

Leukocyte adhesion correlates well with the blood–retinal barrier breakdown in the STZ-induced diabetic rat model. Antibody treatment significantly reduced blood–retinal vascular leakage to 8 ± 1.6 in diabetic animals compared with controls (P < 0.05; n = 8). Administration of the anti–CD49d neutralizing antibody significantly reduced the diabetes-induced vascular leakage to 8 ± 1.6 in diabetic animals compared with controls (P < 0.05; n = 8). **P < 0.05 compared with nondiabetic controls. **P < 0.05 compared with animals treated with the isotype-matched control antibody.

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Intraperitoneal Administration of CD49d Antibody Reduces the Diabetes-Induced Vascular Leakage

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

**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 ± 4.27 in nondiabetic rats (n = 9) to 110.16 ± 25.84 in rats treated with the 5 mg/kg dose of the control antibody (P < 0.05; n = 18) and 95.72 ± 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 ± 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 ± 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.

**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 ± 1.06 in control (nondiabetic) rats to 13.9 ± 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 ± 1.6 in diabetic animals treated with the anti–CD49d antibody compared with 13.9 ± 1.63 in the diabetic animals treated with the control antibody (P < 0.05; n = 8). **P < 0.05 compared with nondiabetic controls. **P < 0.05 compared with animals treated with the isotype-matched control antibody.
in the retina. We next investigated whether VEGF and TNF-α protein levels were affected by the anti-CD49d therapy. In agreement with our previously published results, VEGF and TNF-α levels increased 2 weeks after the onset of diabetes: 11.4 ± 2.2 pg VEGF/mg tissue in the nondiabetic animals compared with 25 ± 5.3 pg VEGF/mg tissue in the diabetic animals and 1.028 ± 0.128 pg TNF-α/mg tissue in the nondiabetic animals compared with 2.24 ± 0.41 pg TNF-α/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 ± 7.4 pg/VEGF/mg tissue in the diabetic animals treated with anti-CD49d compared with 25 ± 5.3 pg VEGF/mg tissue in the diabetic animals treated with the control antibody and 1.42 ± 0.25 pg TNF-α/mg tissue in the diabetic animals treated with the anti-CD49d antibody compared with 2.24 ± 0.41 pg TNF-α/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-κB Activity

Both TNF-α and VEGF gene promoters are under direct stimulatory control from the transcription factor NF-κB. NF-κB activity increases early in the course of diabetes and correlates with both VEGF and TNF-α expression. We next investigated whether CD49d blockade suppresses NF-κB activity in our STZ-induced rodent diabetes model. In agreement with our previously published results, NF-κB 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-κB activity to 1.002 ± 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. Our laboratory has previously demonstrated a role for the ICAM-1/CD18 pair of adhesion molecules in the pathogenesis of early DR. However, ICAM-1 inhibition does not completely abolish leukocyte adhesion, emphasizing the importance of other adhesion molecules in DR. We now report that α4 integrins may play that role. Administration of an anti-α4 integrin antibody attenuated leukocyte adhesion and vascular leakage in our rodent model of DR, suppressed VEGF and TNF-α expression, and reduced NF-κB activity.
VLA-4 (α4β1 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 and by upregulating levels of MCP-1, TNF-α, and VEGF. The reduction of VEGF and TNF-α 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-α, 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-κB (which regulates their gene expression) and the cause of further NF-κB inhibition. Additionally, the reduction in oxidative stress caused by the suppressed leukocyte activation can further reduce the activation of the redox-sensitive NF-κB and the upregulation of VEGF and TNF-α.

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-α) and they are both involved in leukocyte adhesion, it is not fully understood whether these adhesion molecules exhibit 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, can increase VLA-4 affinity for its ligands by inducing a conformational change in the integrin through PI3K. Alternatively, the phosphorylation C pathway, which is activated in diabetes, can promote the high-affinity conformation of α4 integrins through increased calcium and calmodulin activation.

Alternatively, the increased CD49d-mediated leukocyte adhesion in DR may be caused by an upregulation of VLA-4 ligands countered receptors. 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. Cytokines upregulated during the course of DR, such as TNF-α, 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.

Moreover, the neutralizing antibody that we used is raised against α4 integrin (CD49d) and recognizes both the α4β1 complex (VLA-4) and the α4β7 complex. The mucosal adhesion cell adhesion molecule MadCAM-1, which acts as a counterreceptor for the α4β7 ligand and plays an important role in T-lymphocyte homing in the intestine, 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, although its contribution to diabetic retinopathy has not been thoroughly investigated.

In conclusion, we report a role for α4 integrin/CD49d in the pathogenesis of DR. CD49d blockade suppressed the diabetes-induced upregulation of NF-κB activity and VEGF and TNF-α protein levels, and it decreased vascular leakage and leukostasis. CD49d blockade suppresses inflammation and autoimmune diseases. A humanized anti-VLA-4 mAb (natalizumab) has demonstrated clinical activity in relapsing multiple sclerosis, Crohn’s disease, and rheumatoid arthritis, 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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