American Journal of Pathology, Vol. 164, No. 5, May 2004

Simvastatin Inhibits Leukocyte Accumulation and Vascular Permeability in the Retinas of Rats with Streptozotocin-Induced Diabetes

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Leukocytes play important roles in the pathogenesis of diabetic retinopathy. Recently, 3-hydroxy-3-methylglutaryl-coenzyme A reductase inhibitors have been reported to exert various effects in addition to their lipid-lowering ability. We investigated the effects of simvastatin, a 3-hydroxy-3-methylglutaryl-coenzyme A reductase inhibitor, on leukocyte-induced diabetic changes in retinas. Diabetes was induced in Long-Evans rats with streptozotocin, and simvastatin administration was begun immediately after the induction of diabetes. Two weeks of treatment with simvastatin suppressed significantly the number of leukocytes adhering to retinal vessel endothelium and the number of leukocytes accumulated in the retinal tissue by 72.9% and 41.0%, respectively ($P < 0.01$). The expression of intercellular adhesion molecule-1 (ICAM-1) and the CD18 (the common β-chain of ICAM-1 ligands) were both suppressed with simvastatin. The amount of vascular endothelial growth factor in the retina was attenuated in the simvastatin-treated group. To evaluate the effects of simvastatin on leukocyte-induced endothelial cell damage, vascular permeability in the retina was measured with fluorescein-labeled dextran. Treatment with simvastatin markedly reduced retinal permeability ($P = 0.014$). This suggests that simvastatin attenuates leukocyte-endothelial cell interactions and subsequent blood-retinal barrier breakdown via suppression of vascular endothelial growth factor-induced ICAM-1 expression in the diabetic retina. Simvastatin may thus be useful in the prevention of diabetic retinopathy. *(Am J Pathol 2004, 164:1697–1706)*

Retinopathy is a major complication of diabetes and is one of the leading causes of adult blindness in many countries. In diabetic retinopathy, the expression of intercellular adhesion molecule-1 (ICAM-1) on the vascular endothelial cells is up-regulated,[1–3] which leads to leukocyte adhesion to vascular endothelium and to accumulation of leukocytes within the retina.[1,4,5] Leukocytes that are adherent to vascular endothelium have been shown to cause capillary occlusion,[1,4,5] endothelial cell apoptosis,[6,7] and, finally, blood-retinal barrier breakdown (BRB).[1,7–9] These damages result in clinical symptoms of diabetic retinopathy, such as areas of nonperfusion, retinal hemorrhage because of vascular vulnerability, and retinal edema each of which can cause serious loss of visual acuity.

Simvastatin is one of the family of statins, 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase inhibitors, that have been reported to exert many effects in addition to their cholesterol-lowering ability. Recently, statins have been shown to attenuate leukocyte-endothelial cell interactions at sites of inflammation.[10,11] A suggested mechanism for this effect is that simvastatin suppresses the expression of adhesion molecules on endothelium[12–14] through activation of endothelial nitric oxide synthase (eNOS).[12–16] Because ICAM-1-mediated leukocyte-endothelial cell interaction is an important step in the development of diabetic retinopathy, simvastatin should have beneficial effects in the prevention of diabetic retinopathy through suppression of ICAM-1-mediated leukocyte adhesion to vessel walls. Although eNOS is an important mediator for ICAM-1 expression,[2,6,8,17,18] vascular endothelial growth factor (VEGF) is another mediator that up-regulates ICAM-1 expression on endothelial cells[8,19–24] and is an important cytokine in the induction of diabetic retinopathy.[25–30] In that some studies have reported the ability of statins to down-regulate VEGF release,[31–33] VEGF may be the pathway of the effects of statins on diabetic retinopathy.

Although statins have been reported to prevent renal injury,[34–36] to the best of our knowledge, no study has investigated their effects on diabetic retina, other than for their lipid-lowering effect. In the study described herein, we evaluated quantitatively the inhibitory effects of sim-

Supported by a Grant-in-Aid for Scientific Research from the Ministry of Education, Science, and Culture, Tokyo, Japan (to J.K. and Y.H.).

Accepted for publication January 26, 2004.

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vastatin on leukocyte-endothelial cell interactions and BRB breakdown in experimental diabetic retinas. We also investigated the effects of simvastatin on the expression of several important mediators, such as ICAM-1, eNOS, and VEGF, in the diabetic retina. Consequently, the current study estimates the therapeutic efficacy of simvastatin on early diabetic retinopathy.

Materials and Methods

Materials

Male pigmented Long-Evans rats (180 to 200 g, $n = 72$) were purchased from KIWA Laboratory Animals Co. Ltd. (Wakayama, Japan). Each rat received an intraperitoneal injection of streptozotocin (60 mg/kg; Sigma Chemical, St. Louis, MO) in 10 mmol/L citrate buffer (pH 4.5) after an overnight fast. Animals with blood glucose levels greater than 250 mg/dl 24 hours later were considered diabetic. We confirmed that the plasma glucose level in each rat was >250 mg/dl just before the experiment. All rats were kept in an air-conditioned room with a 12-hour light and 12-hour dark cycle and given free access to water and food until they were used for the experiments. Simvastatin was obtained from Merck/Banyu Pharmaceutical (Merck, Rahway, NJ; Banyu, Tokyo, Japan). Simvastatin (0.25, 2.5, and 25 mg/kg/day) was administered orally for 2 weeks from the day of diabetes induction. Animals injected with an equal volume of saline solution alone served as diabetic vehicle-treated controls. All experiments were performed in accordance with the Association for Research in Vision and Ophthalmology (ARVO) Statement for the Use of Animals in Ophthalmic and Vision Research.

Evaluation of Leukocyte Accumulation in Rat Retina

Leukocyte accumulation in retinal microcirculation was evaluated with acridine orange (AO) digital fluorography, which has been described previously in detail elsewhere.[1,5,37,38] Six different rats were used in each group. This technique uses a scanning laser ophthalmoscope (Rodenstock Instruments, Munich, Germany), coupled with a computer-assisted image analysis system, which makes continuous high-resolution images of fundus stained by AO (Wako Pure Chemicals, Osaka, Japan). AO, a metachromatic fluorochrome, is a widely used probe in biochemical and cytochemical studies. The dye emits a green fluorescence when it interacts with DNA. The argon blue laser was used for the illumination source, with a regular emission filter for fluorescein angiography, because the spectral properties of leukocytes stained with AO are similar to those of sodium fluorescein. Immediately before AO digital fluorography, rats were anesthetized with xylazine hydrochloride (4 mg/kg) and ketamine hydrochloride (10 mg/kg) and their pupils were dilated with 0.5% tropicamide and 2.5% phenylephrine hydrochloride. A contact lens was placed on the cornea to maintain transparency throughout the experiments. Arterial blood pressure was monitored with a blood pressure analyzer (IITC, Woodland Hills, CA). Each rat had a catheter inserted into the tail vein and was placed on a movable platform. Body temperature was maintained at between 37°C and 39°C throughout the experiment. AO (0.1% solution in saline) was injected continuously through the catheter for 1 minute at a rate of 1 ml/min. Thirty minutes after the injection, the fundus was observed with the scanning laser ophthalmoscope for evaluation of leukocyte accumulation in the retinal microcirculation. The obtained images were recorded on a S-VHS videotape at the video rate of 30 frames/second for further analysis. Blood was collected to count the number of leukocytes in the peripheral blood by a hematology analyzer (ERMA, Tokyo, Japan) and to measure the serum cholesterol level. After the experiment, each rat was killed with an overdose of anesthesia. The video recordings were analyzed with a computer-assisted image analysis system consisting of a computer equipped with a video digitizer (Radius, San Jose, CA) that digitizes the video image in real time to 640 horizontal and 480 vertical pixels with an intensity resolution of 256 steps. The number of fluorescent dots in the retina within a circle of 1000 μm in diameter from the center of the optic disk was counted as the number of leukocytes accumulated in the retina for each rat.

Evaluation of Leukocyte Adhesion to Retinal Vessels

Leukocyte adhesion to rat retinal vessels was evaluated using the method described elsewhere, with slight modification.[19,39] Six different rats were used in each group. After the induction of deep anesthesia, the chest cavity was carefully opened and a 20-gauge perfusion cannula was introduced into the aorta. Drainage was achieved by opening the right atrium. The animals were then perfused with 100 ml of phosphate-buffered saline (PBS) to wash out blood cells in the vessels, such as erythrocytes and nonadherent leukocytes. After PBS perfusion, the animals were perfused with 25 ml of fluorescein isothiocyanate (FITC)-labeled concanavalin A lectin (ConA; 40 μg/ml in PBS, pH 7.4) (Vector Laboratories, Burlingame, CA). ConA was used to label leukocytes adherent to the vessel walls and vascular endothelial cells. Residual unbound ConA was removed with PBS perfusion. The retina was carefully removed and fixed with 1% paraformaldehyde, and flat mounts were prepared using a fluorescence anti-fading medium (Vector Laboratories). The retinas were then observed using fluorescence microscopy (FITC filter; Olympus Optical, Tokyo, Japan), and the total number of adherent leukocytes per retina was determined.

Semiquantification of eNOS and ICAM-1 Gene Expression with Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR)

Two weeks after the onset of diabetes, one eye from each of six rats in the simvastatin (25 mg/kg/day)-treated, ve-

hicle-treated, and nondiabetic control groups was enucleated. Total RNA was isolated from the retina using TRIzol reagent (Life Technologies, Grand Island, NY). The extracted RNA was quantified, and then 2 μg of the RNA was used to make cDNA with a kit (Omniscript Reverse Transcriptase; Qiagen, Valencia, CA). For semiquantitative PCR, 2 μl of each first-strand reaction was then amplified using eNOS-, ICAM-1-, and β-actin-specific oligonucleotide primers.[40] PCR amplification was performed with the following conditions: denaturation at 94°C for 1 minute, annealing at 60°C for 1 minute, and polymerization at 72°C for 1 minute. The reaction was performed for 32 cycles for eNOS, 34 cycles for ICAM-1, and 29 cycles for β-actin. The primers were GCATGGGCAACTTGAAGAGT (sense) and CTGGGAACCATCCTTTTGA (anti-sense) for eNOS, AGCCTCAGGCCTAAGAGGAC (sense) and AGGGGTCCCAGAGAGGTCTA (anti-sense) for ICAM-1, and GGCATCCTGACCCTGAAGTA (sense) and GCCATCTCTTGCTCGAAGTC (anti-sense) for β-actin. After completion, 10 μl of the reactions were analyzed by agarose gel electrophoresis and ethidium bromide staining to determine the levels of transcript relative to the control transcript β-actin RNA.

Enzyme-Linked Immunosorbent Assay for VEGF and ICAM-1

The animals were killed with an overdose of anesthesia, and the eyes were immediately enucleated. The retina was carefully isolated, placed in 150 μl of lysis buffer (20% glycerol, 10 mmol/L KCl, 1 mmol/L $MgCl_2$, 0.1% Triton, 300 mmol/L NaCl, 0.5 mmol/L dithiothreitol, 0.5 mmol/L phenylmethyl sulfonyl fluoride, 20 mmol/L HEPES, pH 7.9) and sonicated. The lysate was centrifuged at 14,000 rpm for 15 minutes at 4°C, and both the VEGF levels and the ICAM-1 levels in the supernatant were determined with the VEGF kit and the ICAM-1 kit (Quantikine; R&D Systems, Minneapolis, MN), respectively, according to the manufacturer's protocol. Total protein was determined using the bicinchoninic acid kit (Bio-Rad, Hercules, CA) and was used to normalize the VEGF protein levels.

Flow Cytometric Analysis

The surface expression of CD18 on rat leukocytes was determined using flow cytometry using the method described elsewhere with slight modification.[41,42] CD18 was evaluated by direct immunofluorescence staining of whole blood using an established lyse/wash procedure (BD PharMingen, Franklin Lakes, NJ). After the deep anesthesia, the whole blood anti-coagulated with ethylenediaminetetraacetic acid was obtained from each of six rats in the simvastatin (25 mg/kg/day)-treated, vehicle-treated, and nondiabetic control groups. FITC-conjugated mouse (BALB/c) anti-rat CD18 IgG and FITC-conjugated mouse (BALB/c) IgG (as an isotype-matched control) were purchased from BD PharMingen. Immediately after blood collection, 10 μg of FITC-labeled antibody in 100 μl of staining buffer (PBS with 0.1% sodium azide and 1% fetal bovine serum) were incubated with 100 μl of rat whole blood for 30 minutes at room temperature. Erythrocytes were lysed for 15 minutes by addition of 2 ml of 1× fluorescence-activated cell sorting lysing solution (BD PharMingen). The tubes were centrifuged at 200 × *g* for 5 minutes. The cell pellets were resuspended in 2 ml of staining buffer. The tubes were centrifuged at 200 × *g* for 5 minutes and the leukocytes were resuspended in a fixation solution (2% formaldehyde prepared in PBS). Leukocytes were gated by the typical forward and side light scatter profiles. The fluorescence of 10^4 cells was measured on a FACScan (Becton Dickinson, Franklin Lakes, NJ). Leukocytes were gated on the basis of their characteristic forward and side light-scattering properties. The surface expression was presented as the mean channel fluorescence on a logarithmic scale and the percentage of the CD18-positive leukocytes was also evaluated.

Quantification of BRB Breakdown

BRB breakdown was evaluated by measuring the permeability of retina with FITC-conjugated dextran (Sigma-Aldrich, St. Louis, MO) using the method described elsewhere, with slight modification.[19,39] Two weeks after the onset of diabetes, one eye from each of six rats in the simvastatin (25 mg/kg/day)-treated, vehicle-treated, and nondiabetic control groups were used. After deep anesthesia, FITC-conjugated dextran (4.4 kd, 50 mg/ml in PBS, 50 mg/kg body weight; Sigma) was injected intravenously. Ten minutes after injection, the chest cavity was opened and a 20-gauge perfusion cannula was introduced into the aorta. A blood sample was collected immediately before perfusion. After achieving drainage from the right atrium, each rat was perfused with PBS (500 ml/kg body weight) to clear the remaining intravascular dextran. The blood sample was centrifuged at 7000 rpm for 20 minutes at 4°C, and the supernatant was diluted at 1:1000. Immediately after perfusion, the retinas were carefully removed, weighed, and homogenized to extract the FITC-dextran in 0.4 ml of water. The extract was processed through a 30,000-molecular weight filter (Ultrafree-MC; Millipore, Bedford, MA) at 7000 rpm for 90 minutes at 4°C. The fluorescence in each 300-μl sample was measured (excitation, 485 nm; emission, 538 nm) using a spectrofluorometer (Fluor Imager SI; Molecular Dynamics, Sunnyvale, CA) with water as a blank. Corrections were made by subtracting the autofluorescence of retinal tissue from rats without FITC-dextran injection. For normalization, the retinal FITC-dextran amount was divided by the retinal weight and by the concentration of FITC-dextran in the plasma.

Statistical Analysis

All values are expressed as mean ± SEM. The data were analyzed using analysis of variance, with posthoc comparisons tested using the Fisher protected least significant difference procedure. Differences were considered

Table 1. Physiological Variables for Each Study Group

	Systolic BP	Mean BP	Diastolic BP	Heart rate	WBC	BS2	BW1	BW2
Control	118 ± 5	103 ± 4	95 ± 4	294 ± 4	5.0 ± 0.0	191 ± 11		190 ± 5
Vehicle-treated diabetic group	140 ± 10	108 ± 12	92 ± 13	275 ± 6	6.1 ± 0.8	397 ± 53*	194 ± 3	158 ± 12
Simvastatin-treated group	129 ± 7	108 ± 6	98 ± 7	259 ± 5	5.2 ± 0.6	385 ± 20*	196 ± 3	158 ± 14

BP indicates blood pressure, mmHg; total cholesterol, mg/dl; WBC indicates peripheral leukocyte count, 10^9/L; BG indicates blood glucose level, mg/dl; BW1 indicates body weight at the time of streprozotocin injection, grams; BW2 indicates body weight at the experiment, grams; Values are mean ± SEM (the standard margin of error).
*$P < 0.01$ compared with control rats.

statistically significant when the probability values were less than 0.05.

Results

Physiological Data

Table 1 indicates the physiological variables of each group. There were no significant differences among groups in any of the physiological data except for blood glucose levels. The blood glucose level was significantly larger than the control group in both the vehicle-treated and simvastatin-treated groups. No significant difference was found between the vehicle-treated group and the simvastatin-treated group in blood glucose levels.

Leukocyte Accumulation

Immediately after AO was infused intravenously, leukocytes were stained selectively among circulating blood cells. Vascular endothelial cells also were stained. At 30 minutes after AO injection, endothelial cells turned faint because of the wash out effect, and we could identify the accumulated leukocytes in the retina as distinct fluorescent dots with the highest contrast (Figure 1). Figure 2 shows the numbers of leukocytes accumulated in the retina in each group. Few leukocytes were found in nondiabetic control retina. In the vehicle-treated diabetic group, however, accumulated leukocytes increased to 4.7-fold of the control group (58.2 ± 10.8 cells/eye). In the simvastatin (2.5 mg/kg/day)-treated group and sim-

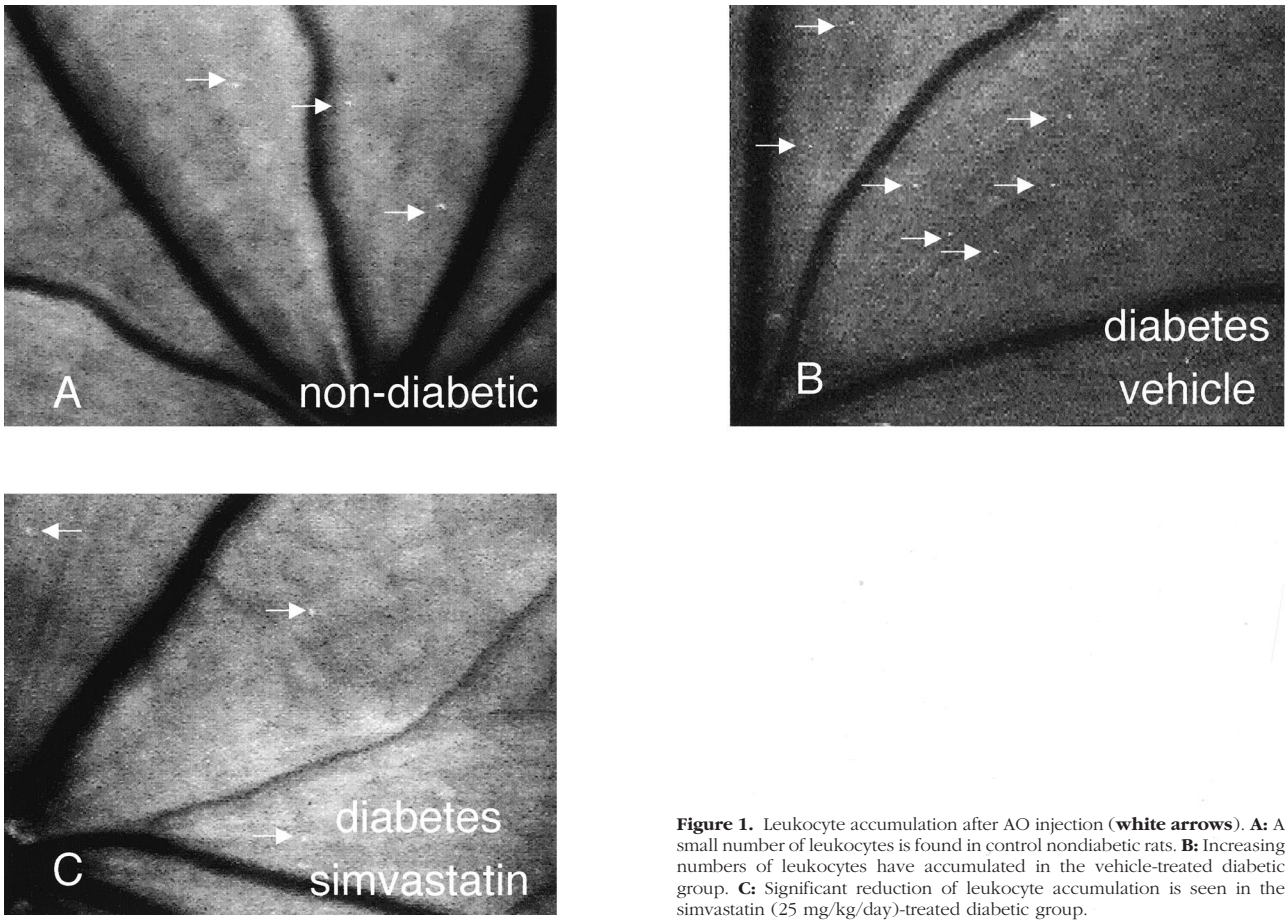

Figure 1. Leukocyte accumulation after AO injection (**white arrows**). **A:** A small number of leukocytes is found in control nondiabetic rats. **B:** Increasing numbers of leukocytes have accumulated in the vehicle-treated diabetic group. **C:** Significant reduction of leukocyte accumulation is seen in the simvastatin (25 mg/kg/day)-treated diabetic group.

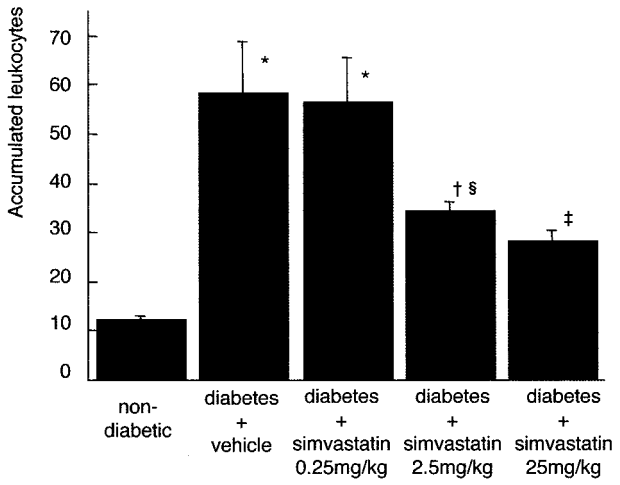

Figure 2. The number of accumulated leukocytes in the retina. Values are mean ± SEM. *, $P < 0.01$; †, $P < 0.05$ compared with control rats. §, $P < 0.05$; ‡, $P < 0.01$ compared with vehicle-treated diabetic rats.

vastatin (25 mg/kg/day)-treated group, the number of accumulated leukocytes were reduced by 41.0% ($P = 0.0152$) and 51.3% ($P = 0.0032$), respectively, as compared with the vehicle-treated group. In the simvastatin (0.25 mg/kg/day)-treated group, there was no significant reduction in the number of the accumulated leukocytes compared with the vehicle-treated group ($P = 0.86$).

Leukocyte Adhesion

In the retinal flat mount, we could observe the leukocytes adhered to vessel walls and retinal endothelium labeled with FITC-coupled ConA because nonadherent leukocytes were removed by perfusion (Figure 3). Few adherent leukocytes were observed in the control group (Figure 3A), whereas the number of adherent leukocytes increased to 3.8-fold of the control group (21.5 ± 2.2 cells/eye) in the vehicle-treated diabetic group (Figure 3B). In the simvastatin (2.5 mg/kg/day)-treated group and simvastatin (25 mg/kg/day)-treated group, adherent leukocytes decreased to 27.1% ($P < 0.0001$) and 55.0% ($P = 0.0016$), respectively, of the vehicle-treated group (Figure 4). Similar changes were observed when adherent leukocytes were evaluated in arteries ($P = 0.013$ and $P = 0.0003$) and veins ($P = 0.036$ and $P = 0.0013$). In the simvastatin (0.25 mg/kg/day)-treated group, there was no significant decrease of the adherent leukocytes compared with the vehicle-treated group ($P = 0.50$).

ICAM-1 Gene Expression and Protein Levels

The levels of gene expression were shown as a ratio to the average values of nondiabetic control rats (Figure 5). ICAM-1 mRNA expression in the vehicle-treated diabetic group was up-regulated to 3.7-fold of the control, nondiabetic group, and was significantly suppressed in the

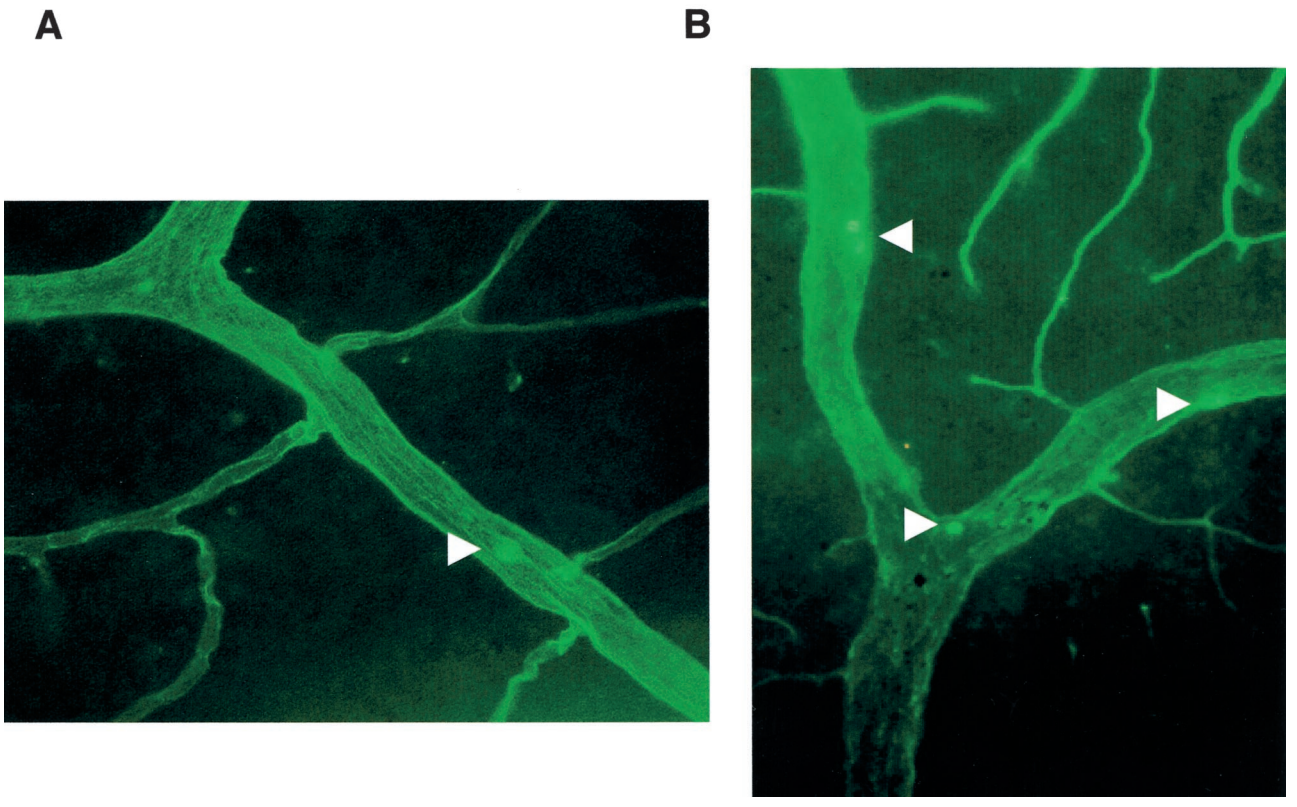

Figure 3. A fluorescence microscopic image of whole-mounted diabetic retina stained with FITC-labeled concanavalin A shows adherent leukocytes (**white arrowheads**). **A:** A small number of leukocytes is found in the control nondiabetic group. **B:** Increasing numbers of leukocytes have adhered in the vehicle-treated diabetic group.

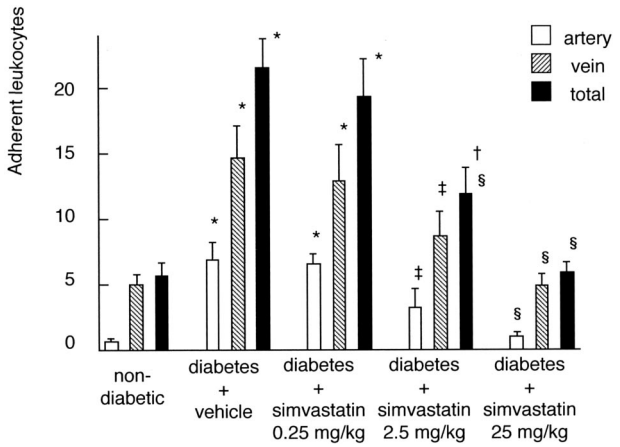

Figure 4. The number of adherent leukocytes in the retinal arteries (white), veins (gray), and total vessels (black). Values are mean ± SEM. *, $P < 0.01$; †, $P < 0.05$ compared with control rats. §, $P < 0.05$; ‡, $P < 0.01$ compared with vehicle-treated diabetic rats.

simvastatin (25 mg/kg/day)-treated group ($P = 0.0046$). This finding was substantiated by enzyme-linked immunosorbent assay (Figure 6A). The ICAM-1 protein levels in the vehicle-treated diabetic group were up-regulated to 1.4-fold of the control nondiabetic group (89.7 + 8.0 pg/mg). Simvastatin (25 mg/kg/day) significantly reduced the ICAM-1 protein levels when compared to the vehicle-treated diabetic group ($P = 0.045$). The eNOS mRNA expression in the vehicle-treated diabetic group was up-regulated to threefold of the control, nondiabetic group, but there was no significant difference between the vehicle-treated diabetic group and the simvastatin (25 mg/kg/day)-treated group ($P = 0.49$).

CD18 Expression on the Leukocytes

CD18 is the common β-chain of ICAM-1 ligands on the leukocytes; LFA-1 (lymphocyte function-associated antigen, CD11a/CD18), Mac-1 (leukocyte integrin, CD11b/CD18), and p150/95 (CD11c/CD18).[43] The histogram of the flow cytometric analysis of CD18 expression on leukocytes is shown in Figure 7 and the statistical data are

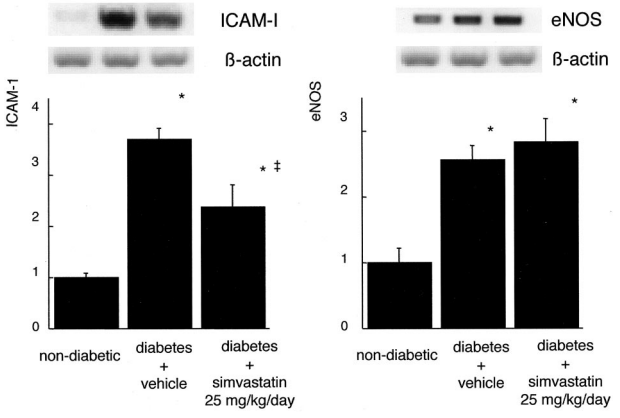

Figure 5. Gene expression of ICAM-1 and eNOS. The levels of gene expression are shown as a ratio to the average value of control rats. Values are mean ± SEM. *, $P < 0.01$ compared with control rats. ‡, $P < 0.01$ compared with vehicle-treated rats.

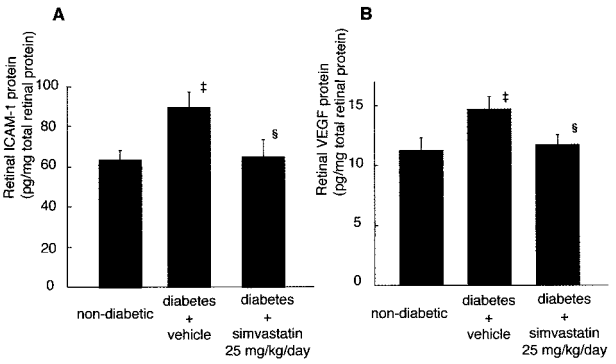

Figure 6. ICAM-1 (**A**) and VEGF (**B**) protein levels in the retina were quantitatively measured using ELISA technique. Values are mean ± SEM. †, $P < 0.05$ compared with control rats. §, $P < 0.05$ compared with vehicle-treated diabetic rats.

shown in Table 2. As evidenced by the increases in mean channel fluorescence, leukocyte CD18 levels of the vehicle-treated diabetic group were 2.4-fold that of the control nondiabetic group and were significantly reduced in the simvastatin-treated group ($P < 0.0001$). In addition, the percentage of the CD18-positive leukocytes in the vehicle-treated diabetic group significantly increased to 1.7-fold of the control nondiabetic group and was significantly decreased in the vehicle-treated diabetic group ($P < 0.0001$).

VEGF Protein Levels

VEGF increases retinal ICAM-1 expression and leukocyte adhesion in addition to its potent vasopermeability function. The VEGF protein levels in the retina were evaluated by enzyme-linked immunosorbent assay (Figure 6B). The VEGF protein levels in the vehicle-treated diabetic group were up-regulated to 1.3-fold of the control nondiabetic group (14.7 ± 1.0 pg/mg). Simvastatin (25 mg/kg/day) significantly reduced the VEGF protein levels when compared to the vehicle-treated diabetic group ($P = 0.049$).

BRB Breakdown

The BRB breakdown is the main factor in the formation of retinal edema, which is one of the most major causes of visual acuity loss in diabetic retinopathy. Figure 8 shows retinal vascular permeability in each group. The levels of BRB breakdown were shown as a ratio to the average values of nondiabetic control rats. Retinal vascular permeability in the vehicle-treated diabetic group increased to 4.1-fold of the nondiabetic group. Treatment with simvastatin (25 mg/kg/day) significantly suppressed the vascular permeability ($P = 0.014$).

Discussion

In this study, we have demonstrated that simvastatin can suppress BRB breakdown in diabetic retina by inhibiting leukocyte-endothelial cell interactions. In addition, our RT-PCR experiments showed suppressed expression of

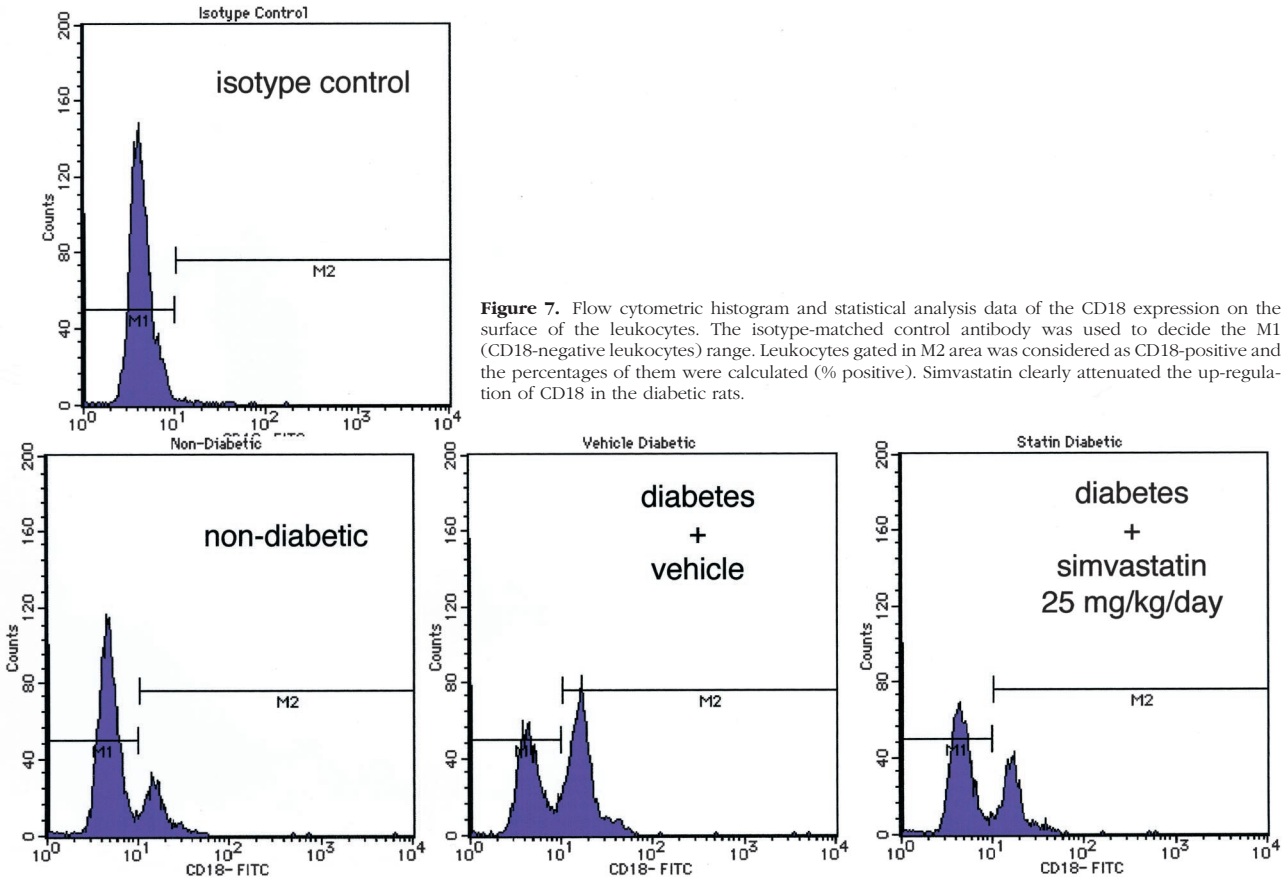

Figure 7. Flow cytometric histogram and statistical analysis data of the CD18 expression on the surface of the leukocytes. The isotype-matched control antibody was used to decide the M1 (CD18-negative leukocytes) range. Leukocytes gated in M2 area was considered as CD18-positive and the percentages of them were calculated (% positive). Simvastatin clearly attenuated the up-regulation of CD18 in the diabetic rats.

ICAM-1, an important adhesion molecule that mediates leukocyte-endothelial cell interactions, in the simvastatin-treated diabetic retina. From these findings, we can suggest that simvastatin inhibits leukocyte-endothelial cell interactions by suppressing ICAM-1 expression on endothelial cells, and thereby prevents any subsequent retinal vascular damage and BRB breakdown in the diabetic retina. Clinically, Gordon and colleagues[44] and Sen and colleagues[45]have reported that simvastatin retards the progression of diabetic retinopathy with concomitant hypercholesterolemia through its cholesterol-lowering ability. To the best of our knowledge, the current study is the first report of the potential of statins to lessen diabetic retinopathy by effects other than lipid lowering.

Table 2. Statistical Data of CD18 Expression

	Mean channel fluorescence	% Positive
Control	8.0 ± 0.4	18.8 ± 0.4
Vehicle-treated diabetic group	13.3 ± 0.6*	44.5 ± 2.8*
Simvastatin-treated group	9.0 ± 0.7†	30.5 ± 4.7*†
P value	<0.0001	<0.0001

The surface expression was presented as the mean channel fluorescence on a logarithmic scale. % Positive indicates the percentage of the CD18-positive leukocytes (decided in Fig. 7). Values are mean ± SEM (the standard margin of error).

*$P < 0.01$ compared with control rats.

†$P < 0.01$ compared with vehicle-treated diabetic rats.

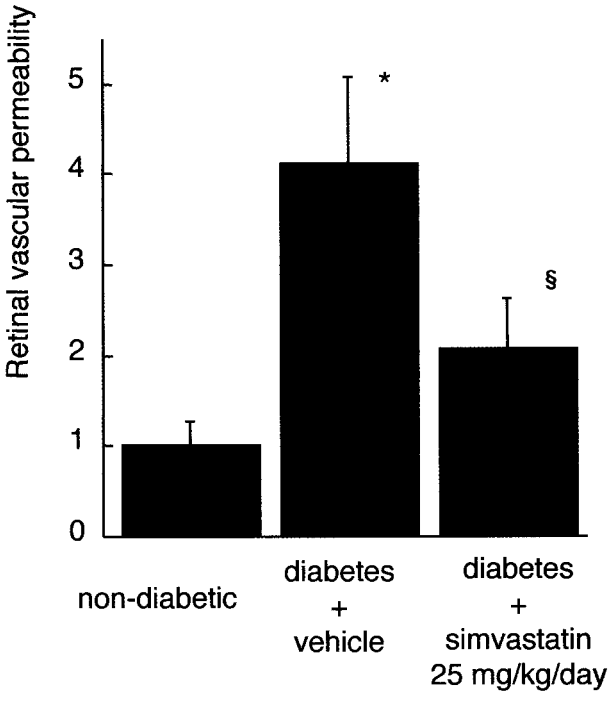

Figure 8. Retinal leakage of FITC-conjugated dextran. The levels of retinal leakage are shown as a ratio to the average value of the control rats. Values are means ±SEM. *, $P < 0.01$ compared with control rats. §, $P < 0.05$ compared with vehicle-treated rats.

ICAM-1 on vascular endothelial cells is the key factor for leukocyte adhesion to the endothelium and for leukocyte migration into the tissue,[46] which causes endothelial cell injury followed by increased retinal vascular permeability, retinal edema, and loss of visual acuity.[6,7,9] It has been reported that ICAM-1 expression is up-regulated in diabetic retina, and is the main cause of leukocyte-endothelial cell interaction.[1–3] Therefore, the ability of statins to inhibit ICAM-1 expression indicates that statins can attenuate leukocyte-endothelial cell interactions and the subsequent endothelial damage and tissue injury. Indeed, many studies have reported that statins can inhibit leukocyte-endothelial cell interactions in many conditions, such as thrombin-activated leukocyte-endothelial cell interactions,[47] angiotensin II-induced injury,[35,48] transient ischemic injury,[36,49] and *Staphylococcus aureus* α-toxin-induced inflammation.[14] These results provide the best evidence to support the view that statins can inhibit leukocyte-endothelial cell interactions independent of their lipid-lowering effect. Consequently, our data are consistent with these results and are the first evidence of statins' effects on diabetic retina.

Moreover, the present study showed that statins suppressed CD18 expression on the diabetic leukocytes. CD18 is also an important molecule for leukocytes-endothelial cell interactions.[43] Statins would show their suppressive effects on leukocyte-induced tissue damage in diabetic rats partly through inhibition of CD18 expression. To our knowledge, the current study is the first to reveal that simvastatin suppresses the CD18 expression on the leukocytes in diabetic rats.

Many studies have reported that eNOS is up-regulated by statins to suppress ICAM-1 expression and after leukocyte-endothelial cell interactions.[13,15,16,49] However, the present study did not show any significant difference in eNOS expression in the diabetic retina between the simvastatin-treated group and the vehicle-treated group. In contrast to the fact that a decrease in NO causes tissue injury in various diabetic organs,[50–52] it has been shown that eNOS expression is up-regulated in diabetic retina[2,6,8,53,54] and that attenuation of NO production can prevent diabetic damage in retinas.[2,6,8] Our present study also showed up-regulation of eNOS in diabetic retinas. In view of the fact that a number of studies have shown that NO can prevent tissue injury by suppressing endothelial expression of adhesion molecules and after leukocyte-endothelial interactions,[17,18,55,56] NO in the diabetic retina might have a protective effect against leukocyte-induced endothelial damage by suppressing ICAM-1 expression in addition to its damaging effect on retinal tissue. In diabetic retinas, the suppression of ICAM-1 by simvastatin might be independent of eNOS expression, although results of our RT-PCR experiments with whole retinas may not reflect local concentrations of NO around the vessels.

VEGF also has been reported to play crucial roles in diabetic retinopathy. VEGF up-regulates ICAM-1 expression and stimulates leukocyte activation and migration.[8,19–24] Our findings of VEGF reduction in simvastatin-treated rats suggest that statins suppress ICAM-1 expression through attenuating VEGF expression rather than NO production. Feleszko and colleagues[33] have also reported that statins suppress VEGF production by tumor cells. Furthermore, Weis and colleagues[31]have reported that endothelial release of VEGF was significantly decreased with high concentrations of statins but not with low-dose statins. Their findings are consistent with our results that high-dose simvastatin can have a beneficial effect on diabetic retinopathy, whereas low-dose simvastatin does not. In addition to its effects on ICAM-1 expression, VEGF would have direct effects on the vasculature increase in permeability. Because VEGF-induced vascular permeability is dependent primarily on ICAM-1-mediated leukocyte stasis in the retina,[21] simvastatin would attenuate the retinal vascular permeability in diabetes by suppressing ICAM-1 meditated leukocyte-endothelial interactions in addition to by decreasing VEGF per se.

Although the clinical dosage of simvastatin is 0.1 ~ 0.2 mg/kg in humans, various dosages from 0.5 mg/kg to 100 mg/kg have been used in experimental models to exert the beneficial effects of statins.[33–36,47–49] These differences may derive from the diversity of the mechanisms of each model or duration of the drug administration. In addition, many studies with streptozotocin-induced diabetic animals were performed for a relatively short duration compared to chronic diabetes in human. Because diabetic changes in patients occur after the duration of the high blood glucose for many years, there would be preceding stages before the onset of the clinical symptoms. Accordingly, the retina with streptozotocin-induced diabetes is regarded to represent the pathogenesis of the early diabetic changes.

From a clinical point of view, leukocytes that are adherent to endothelium do cause endothelial cell injury, BRB breakdown, and capillary occlusion, all of which lead to bleeding, elevated vascular permeability, and the formation of nonperfused areas. Leukocytes that have accumulated in the retina injure the tissue directly through inflammatory reactions or by the formation of hard exudate that disrupt retinal transparency. The increase in vascular permeability produces clinical retinal edema and, when it involves the macula, can lead to loss of visual acuity. These are relatively early features of diabetic retinopathy, but changes that are important to treat to suppress the progression of diabetic retinopathy before the uncontrollable proliferative stage begins. We have shown that simvastatin can inhibit leukocyte-endothelial cell interactions and vascular permeability in the diabetic retina, independent of its cholesterol-lowering ability. Our results thus suggest that statins may be useful for the treatment of early diabetic retinopathy.

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