

Autoantibodies in Type 2 Diabetes Induce Stress Fiber Formation and Apoptosis in Endothelial Cells

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Context: Macular edema contributes to visual impairment, and albuminuria is associated with increased cardiovascular mortality in adults with type 2 diabetes mellitus. These microvascular complications result from increased capillary leakage of plasma proteins whose causation is not completely understood.

Objective: The objective of the present study was to test whether plasma from type 2 diabetes with maculopathy/albuminuria or control subjects contains autoantibodies that can induce apoptosis or activate Rho kinase (ROCK) in endothelial cells.

Design: A cohort of Veterans Affairs Diabetes Trial adults (>40 yr of age) was randomized to standard vs. intensive glycemic treatment lasting 5–7.5 yr.

Setting: The study was conducted in outpatient clinics.

Patients: Case and age-matched control subjects who differed for the baseline presence of significant diabetic maculopathy and/or progression to macro-albuminuria were included in the study.

Intervention: Pharmacological and lifestyle interventions in the Veterans Affairs Diabetes Trial generally resulted in substantially improved glycemic, blood pressure, and lipid levels.

Results: Autoantibodies from patients with macular edema or progression to albuminuria potently induced caspase-dependent apoptosis in endothelial cells (up to 60%), whereas IgG from age-matched normal plasma caused much less apoptosis (<10%; $P < 0.0001$). The active inhibitory autoantibodies triggered stress fiber formation in endothelial cells likely through the activation of Rho guanosine 5'-triphosphatase, which could be nearly completely inhibited by 10 μM Y27632, a specific ROCK inhibitor.

Conclusions: These results suggest that autoantibodies from a subset of advanced type 2 diabetes may contribute to diabetic vascular complications by activating ROCK, inducing stress fiber formation and apoptosis in endothelial cells. (*J Clin Endocrinol Metab* 94: 2171–2177, 2009)

Retinopathy and renal dysfunction frequently coexist in patients with advanced type 2 diabetes (1, 2). Deckert *et al.* (3) and others (4) have suggested that widespread vascular damage resulting in increased capillary permeability to albumin contributes to certain end-organ damage in diabetes. Long-standing hyperglycemia (5) and/or hypertension (6) is a central risk factor in the development of retinal or renal microvascular complica-

tions. The mechanism(s) underlying a particularly strong association between albuminuria and vision-threatening macular edema in patients with type 2 diabetes is still not clear (7).

Growth factors play an important role in the renal or retinal complications of diabetes. Among them, vascular endothelial growth factor induces new blood vessel growth and may increase vascular permeability (8), whereas basic fibroblast growth factor

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Abbreviations: AMD, Age-related macular degeneration; bFGF, basic fibroblast growth factor; DIC, differential interference contrast; DM, diabetes mellitus; F, filamentous; HSPG, heparan sulfate proteoglycan; PI, propidium iodide; RBD, Rho-binding domain; ROCK, Rho kinase; VADT, Veterans Affairs Diabetes Trial.

(bFGF) acts as a potent angiogenesis factor (9) whose levels increase in plasma in adult type 2 diabetes with microalbuminuria or albuminuria (10). In prior work we found significant associations between low-baseline plasma bFGF immunoreactivity and endothelial cell inhibitory autoantibodies, and between the autoantibodies and the need for laser treatment in adults with advanced type 2 diabetes (11, 12). The autoantibodies from adults in the Veterans Affairs Diabetes Trial (VADT) appeared to have a strong inhibitory effect on endothelial cells. In the present study, we tested autoantibodies from subsets of diabetes with maculopathy and/or nephropathy or control subjects for their effects on stress fiber formation or apoptosis in endothelial cells.

Subjects and Methods

Subjects

The baseline clinical characteristics in diabetic subjects from the VADT with maculopathy and nephropathy, maculopathy, or no significant retinopathy are shown in Table 1. We had limited quantities of plasma in subjects with maculopathy. For this reason, potent inhibitory autoantibodies from two additional subjects (patients 1 and 2) not included in Table 1 were used in some experiments. Patient 1 had no maculopathy and did not progress to macro-albuminuria during 4 yr VADT follow-up. Patient 2 progressed from microalbuminuria to overt albuminuria during 4 yr follow-up in the VADT. Subjects were excluded from participation in the VADT if their baseline serum creatinine was more than 1.5 mg/dl.

Diagnostic subgroups

Nonischemic cardiomyopathy

Patient 3. A 55-yr-old male with type 2 diabetes mellitus (DM) for 20 yr and clinically significant macular edema requiring two focal laser treatments was examined. Other complications included depression, mono-neuritis multiplex, renal insufficiency, and progression to albuminuria. Before the onset of refractory paroxysmal atrial fibrillation and hard syncope that required the implantation of an automated defibrillator, plasma demonstrated titers of potent inhibitory endothelial cell autoantibodies that subsequently persisted for at least 2 yr. Serial echocardi-

ography and left cardiac catheterization showed normal coronaries, mild left atrial enlargement, grade I-II diastolic dysfunction, and left ventricular hypertrophy.

Patient 4. A 67-yr-old male with type 2 DM for 7 yr with dry age-related macular degeneration (AMD), mild nonproliferative diabetic retinopathy, and cataracts was examined. Diabetic-related complications included: depression, painful radiculopathy, and albuminuria without renal insufficiency. Plasma demonstrated high titers of very potent endothelial cell inhibitory autoantibodies 1 yr before the first of five hospitalizations over a 10-month period requiring treatment for moderately severe aortic regurgitation, moderate pericardial effusion, pulmonary hypertension, and hypertrophic cardiomyopathy. Other significant findings included: proximal aneurysmal dilatations of the left anterior descending, left circumflex, and right coronary arteries; bradyarrhythmias with hypotension requiring placement of a permanent pacemaker; and hematochezia from an unknown source requiring multiple blood transfusions. Anti-nuclear antibody and erythrocyte sedimentation rate were both within normal limits. Two years after initial presentation with cardiac symptoms, the patient's symptoms resolved in association with the complete disappearance of endothelial cell inhibitory autoantibodies.

Recurrent macular edema

Patient 5. A 58-yr-old male with type 2 DM for 10–15 yr was examined. Family history was significant for the mother with diabetes and end-stage renal disease. Diabetes-related complications include: hypertension and painful peripheral neuropathy. Over a 58-month follow-up period, the patient underwent five focal laser treatments for recurrent macular edema in the right eye. He lost eight lines of letters on the 20/20 Snellen visual acuity chart and was unable to drive a car at night. He experienced a doubling in serum creatinine concentration compared with normal baseline level, progression to macro-albuminuria, and one episode of a transient ischemic attack. Plasma demonstrated the stable presence of inhibitory endothelial cell autoantibodies over the same time period.

Plasma samples

All plasma samples were obtained after subjects provided their consent for participation in an institutional review board-approved study. Archived, coded EDTA plasma samples were kept frozen (-70°C) for up to 4 yr before assay of protein-A eluate fractions for bioactivity in endothelial cells. Bioactivity in protein-A eluate fractions from sera was previously shown to be stable for 5 yr or longer at -20°C (13). Endo-

TABLE 1. Baseline characteristics in study subjects

Patients	Diabetes duration (yr)	HbA _{1c} (%)	ACR (mg/g)	T (yr)	Laser events	S creat (mg/dl)
Maculopathy, nephropathy (n = 4) 64 ± 6 yr Male	12 ± 3	9.9 ± 0.5	523 ± 403 ^{a,b}	3	2.3 ± 1.7 ^a	1.1 ± 0.2, 1.9 ± 0.2 ^{a,c}
Maculopathy (n = 4) 64 ± 5 yr Male	14 ± 5	9.4 ± 1.0	63 ± 59	3	1.3 ± 1.0 ^a	0.9 ± 0.2, 0.9 ± 0.3
Minimal or no retinopathy (n = 9) 57 ± 11 yr Male	9 ± 6	9.8 ± 1.7	38 ± 44	3	0 ± 0	1.0 ± 0.2, 1.0 ± 0.2
Normal subjects (n = 6) 64 ± 19 yr Male		NT	NT			1.0 ± 0.2

Results are mean ± sd. ACR, Albumin to creatinine ratio; HbA_{1c}, glycosylated hemoglobin; NT, not tested; S creat, serum creatinine; T, time interval.

^a $P \leq 0.01$ compared with minimal or no retinopathy.

^b $P = 0.06$ compared with the maculopathy group.

^c $P < 0.05$ compared with the maculopathy group.

thelial cell inhibitory activity in the protein-A eluate fractions from plasma was stable after storage at 0–4 C for 6 months or longer.

Protein-A affinity chromatography

Protein-A affinity chromatography was performed as previously described (13). Briefly, aliquots of plasma were adjusted to pH 8.0 by adding 100 mmol/liter Tris (pH 8). After syringe filtration to clarify samples, 1 ml was applied to a 1-ml column of packed pre-equilibrated protein-A beads (Pierce, Rockford, IL). After washing, the column was eluted. The eluate fractions containing nearly all the recovered protein were pH neutralized and stored at 0–4 C. Inhibitory activity in protein-A eluate fractions was unchanged, appearing in the retentate fraction after dialysis [10 mmol/liter phosphate (pH 7.4)] and ultrafiltration on a 10-kDa cutoff membrane (Centricon-10; Millipore Corp., Bedford, MA). All fractions were sterile filtered before use. Protein concentrations were determined by a bicinchoninic acid protein assay kit (Pierce).

Cell culture and growth assays

Bovine pulmonary artery endothelial cells (Clonetics, Inc., San Diego, CA) were maintained at 37 C in 5% CO₂/95% air in endothelial cell growth medium (Clonetics) plus 10% fetal bovine serum. The cells were passaged continuously and used between passages 4 and 10.

Colorimetric estimation of endothelial cell number

Colorimetric estimation of cell number was performed as previously reported (13). Confluent cells were trypsinized and plated at 10³–10⁴ cells per well in Medium 199 plus 10% fetal calf serum in 96-well plates. After up to 4 d incubation for cells to reach 60–80% confluency, 4 μ l test fractions (final concentration, 30 μ g/ml) were added to wells in quadruplicate. After 2 d incubation in the presence of test fractions, cells were washed with PBS and processed for the colorimetric estimation of cell number, *i.e.* cell-associated acid phosphatase activity, as previously described (13). There was a linear relationship between endothelial cell number and OD at 410 nm. Growth-promoting activity is expressed as a percentage of the control cell number for cells grown in the absence of test protein-A eluate fractions. Each point represents the mean of quadruplicate determinations. The intraassay and interassay coefficients of variation were 4 and 7%, respectively, for 30 μ g/ml protein-A eluted fractions from the plasma of three diabetic subjects ($n = 3$ assays in each patient).

Microscopy imaging

For bright-field images, the approximate 90% confluent endothelial cells in 24-well plates with various treatments were visualized under the Zeiss Axiovert 200 inverted microscope with a phase contrast 20 \times dry lens (NA 0.4; Carl Zeiss MicroImaging, Inc., Thornwood, NY). Alternatively, the cells cultured in glass-bottom dishes were monitored under transmission light mode [differential interference contrast (DIC) images] with a Zeiss LSM510 META confocal microscope with a 63 \times , water immersion lens (NA 1.2). The live cell DIC images of the endothelial cells were captured continuously up to 15 min at room temperature at the speed of about one frame per minute. For fluorescence images the cells were cultured on glass-bottom dishes for 2 d before the experiments. At an indicated time point after addition of autoantibodies, 10 μ M Hoechst dye 33342 (Sigma-Aldrich Corp., St. Louis, MO) was added into the culture medium for 15 min at 37 C in a dark chamber. As described previously, the chromatin condensation and fragmentation was visualized with the Zeiss confocal laser-scanning microscope using a 63 \times water immersion objective, with the excitation wavelength set at 351 nm and the emission filter set at 385–470 nm (14, 15). We used Texas Red-labeled phalloidin to visualize actin stress fibers. After incubation with autoantibodies in the culture medium for 20 min, the cells were fixed in 3.7% paraformaldehyde-PBS containing 0.1% Triton X-100 for 10 min at room temperature (pH 7.2). Cells were then washed twice with PBS and subsequently permeabilized with 0.2% Triton X-100 in PBS. Texas Red-labeled phalloidin (5 μ g/ml) was added in blocking solution

(1% BSA-0.1% Triton X-100 in PBS) for 3 h in a dark chamber at room temperature. After the cells were extensively washed with PBS, the coverslips with cultured cells were mounted on slides with a mounting medium (Gel/Mount; Biomedica, Inc., Foster City, CA). Images were captured using the Zeiss confocal microscope with a 543-nm helium-neon laser for excitation and a 560- to 615-nm filter for emission.

Apoptosis assay

Endothelial cells were cultured in 24-well plates to reach approximately 90% confluence. Purified IgG was added directly to cultured medium. At 0, 6, 12, 18, and 24 h, culture media were removed, and the cells were incubated in a balanced salt solution [140 mM NaCl, 2.8 mM KCl, 2 mM CaCl₂, 2 mM MgCl₂, and 10 mM HEPES (pH 7.2)] containing propidium iodide (PI) (25 μ g/ml) and Hoechst 33342 (10 μ M) for 15 min. The cells were examined by the Zeiss Axiovert 200M microscope using $\times 20$ objective (NA 0.4) for phase contrast, fluorescent blue (Hoechst), and red (PI) images. As a membrane permeable dye, Hoechst stains all nuclei with bright blue, whereas cell impermeable PI only stains the nuclei in the dying cells, which lose their membrane integrity. Early apoptotic cells whose membranes are still intact present only blue nuclei, but the initiation of DNA fragmentation results in patched labeling of the nuclei by Hoechst (15). Cells entering the late apoptotic stage have bright-red condensed chromatin or fragmented nuclei that distinguish them from necrotic ones, which have a uniform red color usually with enlarged nuclei. For each experiment a minimum total of 400 cells for each duplicated sample was counted from at least five randomly chosen fields, and the percentage of total apoptotic cells was obtained by adding early and late apoptotic cells together. Each experiment was repeated at least three times.

Detection of activation of caspase-3

Control and treated cells at 12 h were harvested and washed twice with ice-cold PBS, and lysed with modified radioimmunoprecipitation assay buffer [150 mmol/liter NaCl, Tris-Cl (pH 8.0), 1 mmol/liter EGTA, 1% Triton X-100, 0.1% sodium dodecyl sulfate, and 1% sodium deoxycholate] in the presence of a protease inhibitors cocktail (Pierce). The whole cell lysate was separated on a 15% sodium dodecyl sulfate-polyacrylamide gel after heating the samples at 80 C for 5 min. The proteins were transferred to a polyvinylidene difluoride membrane and probed with rabbit monoclonal antileaved caspase-3 antibody (5A1; Cell Signaling Technology, Inc., Danvers, MA). This antibody specifically detects the large fragment (17/19 kDa) of activated caspase-3 resulting from cleavage adjacent to Asp175 but not full length. The protein-antibody complexes were then blotted with a horseradish peroxidase-linked secondary antibody, and the signal was detected on Kodak films (Eastman Kodak Co., Rochester, NY) using a chemiluminescent kit (Pierce).

RhoA activation assay

The endothelial cells were lysed 45 min after treatment with IgGs from patients, normal or saline buffer control in Rho lysis buffer (16, 17). Bacterial-expressed recombinant protein GST-Rho-binding domain (RBD) (containing activated Rho specific binding peptide sequence) was used to perform GST pull-down assays as described. The total cell lysates and GST pull-down complex were resolved by 12.5% SDS-PAGE and immunoblotted with the anti-Rho monoclonal antibody. The GST-RBD construct and RhoA antibody are generous gifts from Dr. Raymond Habas (Robert Wood Johnson Medical School, Piscataway, NJ).

Statistical analysis

Data are analyzed using Origin Pro7 software (OriginLab Corp., Northampton, MA). Values are mean \pm SE or otherwise indicated. Significance was determined by one-way ANOVA. For the comparisons in Table 1 or Fig. 1, significance was determined by the Wilcoxon rank sum or two-sided *t* test, respectively. A value of $P < 0.05$ was used as criterion for statistical significance.

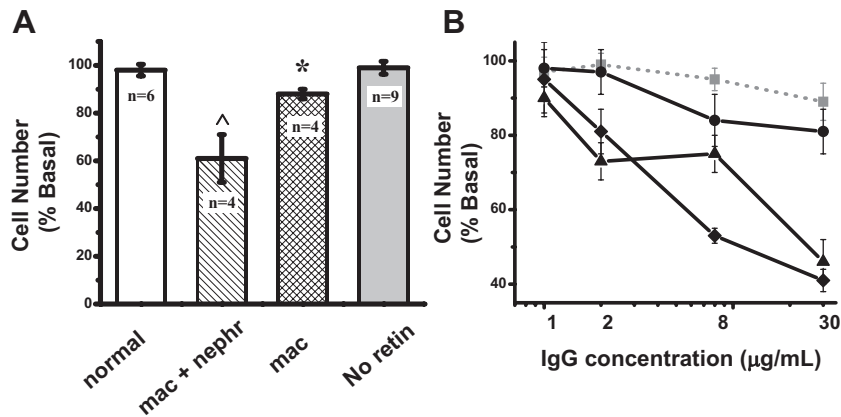


FIG. 1. A, Protein-A eluates from diabetes with maculopathy (mac) and nephropathy (neph) or maculopathy alone causes a significant decrease in cell number compared with eluates from diabetes with no retinopathy (retin) or age-matched normal subjects. A 1:50 dilution (30 μ g/ml) of the protein-A eluate was incubated with cells for 48 h as described in *Subjects and Methods*. \wedge , $P < 0.01$; *, $P < 0.05$. B, Dose-proliferation curves for inhibition of endothelial cell number by the indicated concentrations of protein-A eluate fractions from: a representative patient without maculopathy (square); patient 5 with recurrent macular edema and nephropathy (circle); patient 3 with macular edema, nephropathy, and cardiomyopathy (triangle); and patient 4 with AMD and cardiomyopathy (diamond).

Results

Increased growth-inhibitory activity of protein-A elutes of plasma from subsets of diabetes

Mean protein-A eluted inhibitory activity ($61 \pm 20\%$, $n = 4$) in diabetic subjects with maculopathy and progression to nephropathy significantly exceeded ($P < 0.01$) mean activity in normal subjects ($98 \pm 6\%$, $n = 6$) and in diabetic subjects with minimal or no retinopathy ($99 \pm 8\%$, $n = 9$) (Fig. 1A). It was also significantly more inhibitory ($88 \pm 4\%$, $n = 4$; $P < 0.05$) in diabetic macular edema alone compared with control subjects. To test whether this inhibitory activity comes from autoantibody, we used a 1:50 dilution (30 μ g/ml) of the IgG fraction extracted from plasma using protein-A affinity chromatography.

Titer

Dose-proliferation curves in endothelial cells demonstrated high potency and/or a high titer of inhibitory autoantibodies in plasma from patients with macular edema and other complications. Two diabetic subjects with nephropathy and nonischemic cardiomyopathy (patients 3 and 4) had highest potency inhibitory autoantibodies (Fig. 1B).

Physical and chemical properties of the inhibitory activity in protein-A eluates

Mass spectrometry of active inhibitory protein-A samples from three diabetic maculopathy plasmas demonstrated peaks at 150, 112, 77, 56, and 23 kDa (not illustrated). Inhibitory activity in the protein-A eluates from three diabetic maculopathy plasmas was inactivated 50% by strong heat ($95^\circ\text{C} \times 5$ min) or 23% by exposure to a reducing agent (6 mM dithiothreitol $\times 2$ h at 25°C).

Rho kinase (ROCK)-mediated stress fiber formation and cell contraction in endothelial cells induced by diabetic plasma autoantibodies

When the endothelial cells were exposed to IgG autoantibodies from diabetic plasma but not normal control plasma, the cells

started to contract within 1 min. Ten minutes after exposure to potent diabetic plasma IgG autoantibodies, endothelial cells had retracted from their prior close cell-cell contacts (Fig. 2A). The stress fiber could be observed in DIC images. We used Texas Red-conjugated phalloidin to label further the stress fibers. As shown in Fig. 2B, the cells displayed dramatic filamentous (F)-actin immunoreactive cortical stress fibers. It is well documented that the RhoA/ROCK protein kinase-signaling pathway plays a central role in regulating stress fiber formation and contractile events. We next examined whether RhoA is activated in endothelial cells by addition of autoantibodies. Recombinant protein GST fused with the RBD (GST-RBD) was used to pull down activated Rho but not resting Rho protein. Although the total Rho proteins are the same in all the samples (Fig. 2C, lanes 1–3 for the whole cell lysis), addition of autoantibodies

from diabetic plasma greatly induced RhoA activation in endothelial cells compared with no treatment or treatment with IgG from normal plasma (lane 6 *vs.* lanes 5 and 4). Moreover, the formation of stress fiber could also be largely inhibited by coincubation with a specific ROCK inhibitor, Y27632 (10 μ M) (Fig. 2D).

Caspase-dependent apoptosis induced by diabetic plasma autoantibodies in endothelial cells

Next, we tested whether the widespread cell death induced by diabetic plasma IgG autoantibodies (Fig. 3A) was through the apoptosis pathway. The endothelial cells treated with diabetic plasma IgG displayed condensed nuclear and fragmented DNA, a hallmark of apoptosis (Fig. 3B, indicated by *arrows*). A total of 50% apoptosis occurred in cells exposed (for 12 h) to IgG fractions of plasma from diabetic maculopathy or progression in albuminuria subjects (Fig. 3C). In contrast, only 7.5% cells underwent apoptosis in cells exposed to normal human IgG fractions. Western blotting with antibodies specific for cleaved caspase-3 indicated the activation of caspase-3 in cells exposed to diabetic IgG autoantibodies (Fig. 4A). A broad-spectrum inhibitor of caspases, quinoline-val-asp-difluorophenoxymethylketone, nearly completely blocked apoptosis in cells exposed to diabetic plasma IgG (Fig. 4B), suggesting that the diabetic plasma IgG induced apoptosis through the caspase-dependent pathway. Y27632 (10 μ M) could significantly inhibit endothelial cell apoptosis from diabetic IgG fraction ($n = 3$, Fig. 4C).

Discussion

Endothelial cell binding autoantibodies were reported in type 1 diabetes in association with proliferative diabetic retinopathy (18). However, to our knowledge this is the first demonstration that circulating autoantibodies from type 2 diabetes with macular edema and/or progression to nephropathy cause stress fiber

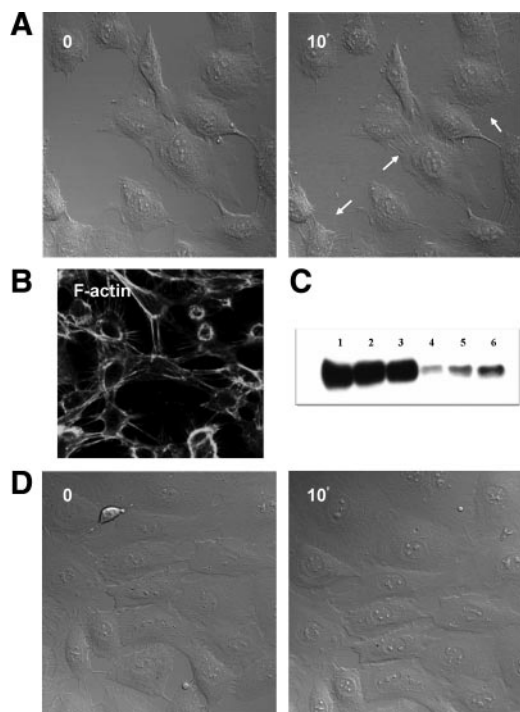


FIG. 2. Autoantibodies induced stress fibers in endothelial cells through the ROCK pathway. **A**, DIC images were recorded every minute after addition of purified IgG from patient no. 2. Images at 0 and 10 min are shown here. The autoantibodies induced stress fiber formation in almost every cell after 10 min (right panel), which is indicated by arrows. **B**, Representative confocal microscopy image of F-actin further revealed the formation of stress fiber. Texas Red-conjugated phalloidin was used to detect F-actin. **C**, Western blot of RhoA protein. Bovine endothelial cells with treatment of IgG from normal control (lanes 2 and 5), patient 5 (lanes 3 and 6), or without treatment (lanes 1 and 4) were lysed, and the GST-RBD pull-down assay was performed. Only activated RhoA appeared in bead pellets in the GST-RBD complex. Lanes 1–3 contain whole cell lysate, and lanes 4–6 have GST-RBD pull-down. **D**, The endothelial cells were preincubated with 10 μM Y27632, a ROCK inhibitor, for 15 min before addition of purified IgG from patient 5. Y27632 blocked the formation of stress fiber.

formation and apoptosis in endothelial cells. These findings may be consistent in part with a report that IgG autoantibodies in lupus patients with nephropathy induced apoptosis in endothelial cells (19). The apparent molecular masses, sensitivity to heat, and partial inactivation by thiols are consistent with an active inhibitory component in diabetic maculopathy/albuminuria plasmas that consists of an IgG or its heavy or light chain subunits.

Antiendothelial cell autoantibodies are common in systemic autoimmune diseases such as lupus (20) or vasculitis (21). However, our finding of inhibitory endothelial cell autoantibodies in adult type 2 diabetes is surprising because type 2 diabetes is not an autoimmune disease. The prevalence of endothelial cell inhibitory autoantibodies in patients with advanced type 2 diabetes from the VADT (32%) (11) was similar to that reported in patients with type 1 diabetes of 10 or more years' average duration (22). This suggests that vascular damage *per se* may contribute to the development of IgG endothelial cell inhibitory autoantibodies in advancing type 2 diabetes.

The loss of heparan sulfate proteoglycan (HSPG) from (glomerular) endothelial cells has been proposed as an underlying mechanism in the development of diabetic microalbuminuria (23) and in generalized diabetic vasculopathy (24). HSPG is a

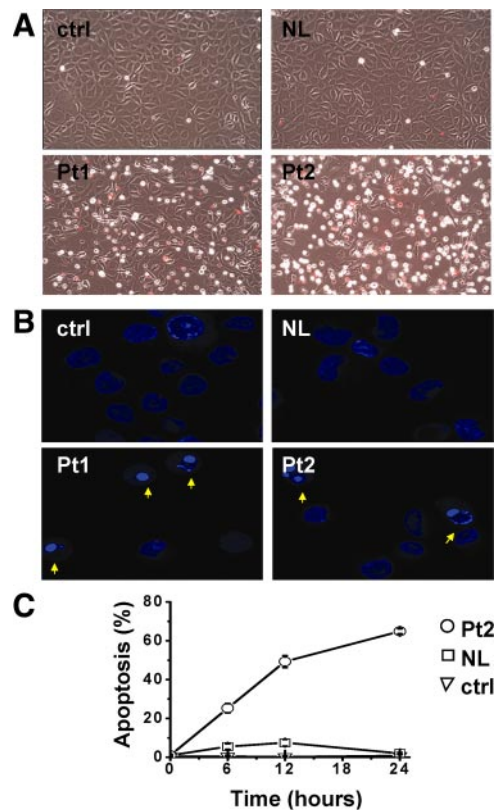


FIG. 3. Autoantibodies from type 2 diabetic patients induced apoptosis in endothelial cells. Bovine endothelial cells were cultured with or without purified IgG from normal or diabetic patients. **A**, Bright-field images of endothelial cells after 12 h treatment with purified IgG from normal or diabetic patients. Without addition of purified human IgG, endothelial cells displayed a rapid growth rate and normal morphology (ctrl). Addition of normal human IgG in the culture medium (NL) slightly inhibited the growth of the cells if any effect at all. However, addition of similar concentrations (20–30 $\mu\text{g}/\text{ml}$) of purified IgG from diabetic patients [patient 1 (Pt1) and patient 2 (Pt2)] induced significant cell death. **B**, Hoechst dye 33342 staining images showed the nuclear fragmentation and condensation that are the hallmarks for apoptosis in cells treated with purified IgG from diabetic patients, but not in control cells or cells treated with normal human IgG. **C**, Time course of IgG-induced apoptosis. Data are from at least three experiments (mean \pm SE). $P < 0.0001$ at 6, 12, and 24 h. Results similar to those in **A** were observed from IgG fraction of the plasma of four diabetic subjects with maculopathy.

low-affinity receptor for bFGF that is abundant on endothelial cells (25). HSPG is also a known target for autoimmunity (26). Autoantibodies that bind to HSPG might account for the association between diabetic plasma inhibitory endothelial cell autoantibodies and low plasma levels of the heparin-binding, bFGF in patients with long-standing diabetes and maculopathy (11).

Apoptosis in photoreceptors is a characteristic feature in some forms of macular degeneration (27). Strong activation of endothelial cell ROCK by diabetic plasma autoantibodies may contribute to disruption in endothelial cell-cell contacts that comprise the blood-retinal barrier (28), thereby contributing to diabetic macular edema or possibly early AMD (observed in two of the eight maculopathy subjects, Table 1). bFGF is a survival factor in mesenchymal-derived cells (29) and photoreceptors (30). Yet, in our preliminary experiments, coincubating endothelial cells with 100 pg/ml recombinant human bFGF did not rescue or prevent apoptosis induced by 4–30 $\mu\text{g}/\text{ml}$ of the active inhibitory protein-A eluate fraction from five of the five diabetic

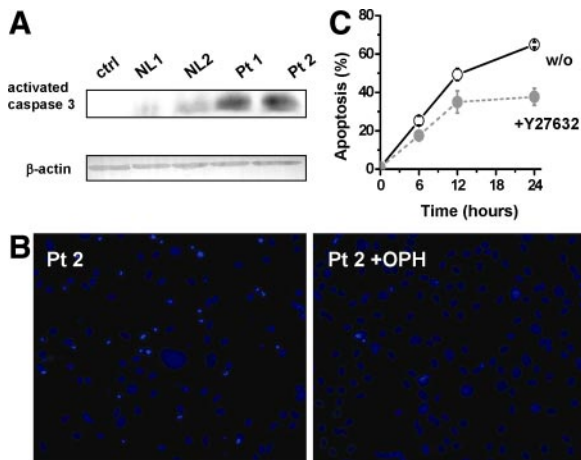


FIG. 4. Inhibition of caspases or ROCK rescued endothelial cells from autoantibodies induced apoptosis. A, Western blot demonstrated that caspase-3 was activated in autoantibody treated endothelial cells. ctrl, Without treatment; NL1, IgG from normal human no. 1; NL2, IgG from normal human no. 2; Pt 1, IgG from diabetic patient no. 1; Pt 2, IgG from diabetic patient no. 2. Mouse monoclonal antibody specifically against cleaved but not full-length caspase-3 was used here. B, Hoechst dye staining images of endothelial cells after 12 h treatment with purified IgG from patient no. 2. A total of 10 mM pan-caspase inhibitor quinoline-val-asp-difluorophenoxymethylketone in the medium almost completely blocked apoptosis in endothelial cells (*lower panel*). Compared with cells without (w/o) OPH, the cells appeared to have normal morphology, and nuclei are intact. C, Time course of apoptosis in endothelial cells treated with antibodies from diabetic patient 2 with or without the presence of Y27632. ROCK inhibitor Y27632 could prevent a significant amount of endothelial cells from autoantibody induced apoptosis. Data are from at least three experiments (mean \pm SE). $P < 0.01$ at 6, 12, and 24 h. Similar results were obtained with autoantibodies from diabetic macular edema patient plasma using the colorimetric cell number assay.

maculopathy plasmas. The average half-maximal inhibitory concentration in endothelial cells from three diabetic protein-A eluates [$(4 \times 10^{-3} \text{ g/liter}) \times (0.7 \times 10^{-5} \text{ mole/g})$], 30 nM, is in the range of the dissociation constant reported for bFGF binding to its low-affinity HSPG receptor (31). This is consistent with HSPG as a possible target for some of the inhibitory autoantibodies.

The mechanism for diabetic plasma autoantibody induced apoptosis in endothelial cells requires further investigation. Morphologically, diabetic autoantibodies induced endothelial cell rounding and detachment from the extracellular matrix suggestive of a process called anoikis, or detachment-associated apoptosis (32). It is possible that IgG autoantibodies or heavy or light fragments may compete with a component of the extracellular matrix, *e.g.* the heparin-binding domain of fibronectin (33), for cell binding to endothelial cell surface HSPG. It was reported that syndecan, a transmembrane HSPG, associates with intracellular actin when it is cross-linked at the cell surface (34). Stress fiber activation in fibroblasts from fibronectin null mice required cell binding via both $\beta 1$ integrin and antisyndecan antibodies deposited in the extracellular matrix (35). More study is needed to determine whether circulating autoantibodies in type 2 DM may cross-link endothelial cell HSPG cell surface receptors, causing activation of the RhoA/ROCK pathway. ROCK-induced cortical stress fibers (Fig. 2B) may contribute to the loss of endothelial cell attachment and apoptosis via several different pathways (36).

Endothelial cell autoantibodies are thought to play a role in Kawasaki's disease, a medium-sized vasculitis of unknown etiology associated with serious late cardiac sequelae in affected children (37). In the present study, patient 4 suffered with a constellation of unusual cardiac manifestations, coronary artery aneurysms, pericardial effusion, aortic regurgitation, and hypertrophic cardiomyopathy, which are not atypical of the carditis found in patients with Kawasaki's disease (38). It is not clear whether increased potency of inhibitory endothelial cell inhibitory autoantibodies from patients 3 and 4 may have accounted in part for these two patients suffering from a wide range of neurological, and nonischemic cardiac complications. Active inhibitory diabetic plasma autoantibodies ($n = 6$) decreased survival and promoted ROCK dependent neurite retraction in differentiated PC-12 pheochromocytoma neuron-like cells (39). A purified fraction of the patient 5 protein-A eluate ($1 \mu\text{g/ml}$) increased intracellular calcium in mouse HL-1 atrial cardiomyocytes. These preliminary data suggest additional possible cellular targets for the autoantibodies.

In summary, our data provide evidence that macular edema in adults with advanced type 2 diabetes may be mediated in part by circulating autoantibodies that cause stress fiber formation in endothelial cells. The baseline presence of autoantibodies was associated with the subsequent need for laser photocoagulation to treat macular edema (11), and in some patients with the development or progression of diabetic nephropathy (Table 1 and Fig. 1A). Autoantibodies may represent a novel pathogenic mechanism for sustained activation of endothelial cell ROCK, perhaps contributing to the progression from microalbuminuria to macro-albuminuria in type 2 DM (40).

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