a-Series Gangliosides Mediate the Effects of Advanced Glycation End Products on Pericyte and Mesangial Cell Proliferation

A Common Mediator for Retinal and Renal Microangiopathy?

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Advanced glycation end products (AGEs) are involved in the development of microvascular complications, including alterations of retinal pericyte and renal mesangial cell growth occurring during diabetic retinopathy and diabetic nephropathy, respectively. Because gangliosides are implicated in the regulation of cell proliferation, we hypothesized that AGEs could exert cellular effects in part by modulating ganglioside levels. Results of the present study indicate that AGEs caused an inhibition of both bovine retinal pericyte (BRP) and rat renal mesangial cell (RMC) proliferation, associated with an increase of a-series gangliosides consecutive to GM3 synthase activity increase and GD3 synthase activity inhibition. Similar modifications were also found in the renal cortex of diabetic db/db mice compared with controls. Treatment of BRP and RMC with exogenous a-series gangliosides decreased proliferation and blockade of a-series gangliosides with specific antibodies partially protecting the two cell types from the AGE-induced proliferation decrease. Further, inhibition of GM3 synthase using specific SiRNA partially reversed the AGE effects on mesangial cell proliferation. These results suggest that a-series gangliosides are mediators of the adverse AGE effects on BRP and RMC proliferation. They also raise the hypothesis of common mechanisms involved in the development of diabetic retinopathy and diabetic nephropathy. Diabetes 54:220–227, 2005

Microangiopathy is a chronic complication of diabetes characterized by structural and functional alterations of microvessels. Retina and kidney are two main targets of the pathology leading to diabetic retinopathy and diabetic nephropathy, respectively. Diabetic retinopathy is the second cause of blindness in developed countries (1). Capillaries undergo progressive structural alterations such as basement membrane thickening, specific loss of pericytes, and subsequent modifications of endothelial cell proliferation and function. Combined with ischemia, these alterations damage microvessel walls and favor excessive capillary permeability, edema, microaneurisms, and hemorrhage, thereby threatening vision (2). Diabetic nephropathy is considered a major cause of mortality in diabetic patients (3). One of the main features of the pathology is glomerular enlargement. This is the consequence of basement membrane thickening and expansion of the mesangium due to hypertrophy of growth-arrested mesangial cells and accumulation of extracellular matrix proteins. Combined with hemodynamic defects, these events trigger glomerular sclerosis, impaired glomerular filtration rate, and microalbuminuria resulting in severe renal insufficiency (4).

On the other hand, although the pathogenic bases of diabetic retinopathy and diabetic nephropathy are not fully understood at cellular and molecular levels, regulation of cell proliferation and cell-cell and cell-matrix interactions seem to play an important role. A number of biochemical hypotheses explaining the mechanisms involved in the development of diabetic microvascular complications have been proposed—among others, the formation of advanced glycation end products (AGEs) (rev. in 5). Reducing sugars such as glucose react nonenzymatically with amino groups of proteins, lipids, and nucleic acids through a series of reactions finally producing AGEs. Glycation is glucose concentration–dependent and is thus enhanced in diabetes. It occurs preferentially with long-lived proteins exposed to blood glucose such as extracellular matrix or circulatory proteins, altering their structure and function. In addition, AGEs can bind membrane receptors to induce cellular responses via genera-
tion of oxidative stress (6,7), activation of nuclear factor κB (5,7), and expression of various genes such as pro-inflammatory cytokines or adhesion molecules (8,9). All these modifications have important biological effects that can explain many of the changes observed in diabetic microvascular complications, including an increase in vascular permeability, increase in extracellular matrix production and stiffness, and alteration in cell-matrix interactions and cell growth (10). Indeed, many in vivo and in vitro studies have pointed out the implication of AGEs in the development of diabetic retinopathy and diabetic nephropathy (10,11).

Gangliosides are glycosphingolipids concentrated in plasma membrane microdomains and characterized by the presence of sialic acid in their structure. Successive sialylations of lactosylceramide lead to monosialoganglioside GM3, disialoganglioside GD3, and trisialoganglioside GT3. These gangliosides are then converted by the sequential actions of glycosyltransferases and sialyltransferases into more complex gangliosides to form the a-, b-, and c-series, respectively (12). Gangliosides are known to play major roles in cell-cell and cell-matrix recognition via interactions with adhesion receptors such as integrins, with matrix proteins, or with other glycosphingolipids. Moreover, gangliosides, and in particular the a-series gangliosides, have been implicated in the regulation of cell proliferation, through modulation of different growth factor activities (13). Because AGEs are implicated in many perturbations of cell growth and viability occurring during diabetic retinopathy and diabetic nephropathy, we hypothesized that gangliosides could participate in mediating their effects. In the present study, we investigated the effect of AGEs on the proliferation of two cell types (retinal pericytes and renal mesangial cells) involved in diabetic retinopathy and diabetic nephropathy, respectively. Results indicated that AGEs cause an inhibition of proliferation of both cell types, at least in part by accumulating the a-series gangliosides. The observations suggest that the a-series gangliosides might represent a common mechanism involved in cellular alterations occurring during the development of diabetic retinopathy and diabetic nephropathy.

**Isolation of mice renal cortex.** Renal cortex fragments were isolated from kidneys of 11-week-old diabetic db/db and control db/+ mice (Charles River). Briefly, animals were anesthetized and killed, and their kidneys were removed. Kidney cortex fragments were then dissected and mechanically homogenized with a Dounce homogenizer in 25 mMol/1 HEPES, 1 mMol/1 EDTA, and 10 μMol/1 protease inhibitors.

**Diabetic preparation and treatment.** AGEs were prepared as previously described (16) by incubation of fatty acid-free and low-endotoxin BSA (7.2 mg/ml final concentration) (Sigma) with 100 mMol/1 methylglyoxal (Sigma) at 37°C for 50 h. BSA in the absence of methylglyoxal was incubated in the same conditions and used as a control preparation (control BSA). AGE or control BSA (3 μMol/1 final concentrations) was added to the culture media 24 h after seeding. Each cell type was treated during one passage (~7 days for BRPs and 4 days for RMCs). Cultured medium was replaced by fresh medium every 2 days.

**Measurement of cell growth.** At the end of the treatment, cells were harvested with trypsin, and cell pellets were washed twice with ice-cold PBS (Sigma). For each sample, an aliquot of cells was counted using a hemocytometer to determine the cell number. Another aliquot was used to measure protein concentrations according to Bradford.

**Ganglioside analysis.** For metabolic labeling of gangliosides, 0.2 or 1 μCi/ml for labeling experiments with high specific activity (GM2 and GM1 measured, respectively) of α-[35S]sialic acid (325.5 mCi/mmol, American Life Sciences, Boston, MA) was added to the media overnight. Cells were then collected by trypsinization and washed three times with PBS. Extraction of gangliosides from cell pellets or cortex homogenates was performed as previously described by Natalizio et al. (17). Briefly, total lipids were extracted with chloroform/methanol (1:1, vol/vol) and submitted to partition with chloroform/methanol/PBS 1 mMol/1 (10:10:1, vol/vol/vol). The upper phases containing gangliosides were next desalted on a C18 silica gel column (Waters Corporation, Milford, MA) and analyzed by high-performance thin-layer chromatography (HPTLC) (Merk, Darmstadt, Germany). Plates were developed in chloroform/methanol/0.2% CaCl2 (55:45:10, vol/vol/vol), and gangliosides were visualized by autoradiography using phosphor screen and Storm 820 (Molecular Dynamics, Amersham Pharmacia Biotech, Piscataway, NJ) and by resorcinol staining (resorcinol 0.3% [Sigma] 0.03% CuSO4, 30% HCl) with Image Master VDS-CL (Amersham Pharmacia Biotech). Quantification was done with Image Quant (Molecular Dynamics). Because GTb1 is absent in both BRP and RMC ganglioside profiles, it was added as an internal standard in the samples before lipid extraction.

**Measurement of GM3 and GD3 synthase activities.** At the end of treatment, cells were washed with PBS, incubated for 20 min at 4°C in 50 μl lysis buffer (20 mMol/1 sodium cacodylate, pH 6.6 [Sigma], 0.2% Triton X-100, 1 mMol/1 EDTA, 10 μMol/1 protease inhibitors [Calbiochem, La Jolla, CA]), and then collected by scraping. Four dishes of BRPs (~3–6 × 105 cells) and three dishes of RMCs (~3–6 × 104 cells) were pooled. Cells lysates were centrifuged at 10,000g for 5 min, and the supernatants in the supernatants were used to measure enzyme activity. Cortex homogenates were centrifuged at 1,000g for 2 min, and the postnuclear supernatant was used to measure enzyme activities. Equal amounts of proteins from each sample (~500 μg for cells and 200–400 μg for cortex) were used to perform the assay. Samples were then mixed with an equal volume of reaction buffer containing at final concentrations the following: 0.1 mMol/1 lactosylceramide or GM3 (Matreya, Biovalley, Marne la Vallée, France) for GM3 and GD3 synthase activities, respectively, and 4 μCi/ml [sialic acid-4,5,6,7,8,9-3H]CMP-sialic acid (325.2 mCi/mmol) (PerkinElmer Life Sciences), 100 μMol/1 CMP-sialic acid (Sigma), 10 mMol/1 MgCl2, 0.2% Triton X-100, and 100 mMol/1 sodium cacodylate, pH 6.6. After shaking, the reaction mixtures were incubated at 37°C for 150 min. Reactions were stopped by loading the samples on silica gel 60 columns (Merck) to separate excess substrates from products. After washing the columns with water, gangliosides were eluted with chloroform/methanol (1:1, vol/vol) and finally separated by thin-layer chromatography (Merk), and reaction products were revealed by autoradiography. Enzyme activities were expressed as picomoles of product formed per hour per milligram proteins.

**Treatment with exogenous gangliosides.** To evaluate the effect of exogenous gangliosides on BRP and RMC proliferation, cells were cultured in 96-well plates. Exogenous glycolipids GM3, GM2, GM1, GD1a, glucosylceramide, and lactosylceramide (Matreya) were added to the culture complete medium at a final concentration of 50 μMol/1 in the form of complexes with BSA at a ratio of 1:1 in DMEM–10 mMol/1 HEPES, pH 7.4. At the end of the treatment, cells were washed twice with PBS and lysed for 30 min at 37°C in GPO buffer (PBS 10 mMol/1, NP40 1% [Pierce, Perbio Science, Brebiers, France], sodium deoxycholate 0.5%, SDS 0.1%, and 10 μMol/1 protease inhibitors). Total proteins were finally measured using the BCA protein assay (Pierce) to assess cell proliferation, because in our experiments, cell number correlated with total protein concentrations (Fig. 1).
Treatment with anti-a-series ganglioside antibodies. To block the potential effects of a-series gangliosides, cells were cultured in 96-well plates and treated with 3 μmol/l AGE or control BSA in the presence or absence of 50 μg/ml polyclonal anti-GM2 (Calbiochem) or anti-GM1 (Matreya) antibodies. Rabbit IgG (Sigma) was used as a control. At the end of the treatment, cells were washed twice with PBS and lysed in 50 μl Ripa lysis buffer, and proteins were measured to assess cell proliferation.

Transfection of RMCs with GM3 synthase SiRNA. To block GM3 synthase activity, RMCs were grown in six-well plates to 30% confluence and transfected with specific GM3 synthase SiRNA designed against a rat cDNA sequence (Ambion, Huntingdon, U.K.) using Oligofectamine reagent (Invitrogen). The GM3 synthase antisense sequence used was GGGUUAUUCUGAAC AUGUUtt. In preliminary experiments, a dose-response study was performed by transfecting cells with increasing concentrations of SiRNA (0 – 800 nmol/l). After 72 h of transfection, GM3 synthase activity was measured on homogenates of transfected cells. Proliferation was next assayed in SiRNA transfected cells. To this end, 24 h after transfection with 400 nmol/l SiRNA, cells were treated with 3 μmol/l of either control BSA or AGE (3 days). Cells were then washed twice with PBS and lysed in 50 μl Ripa lysis buffer, and proteins were measured to assess cell proliferation.

Statistical analysis. Data are expressed as means ± SE and presented as percentage of controls. In cell studies, the Wilcoxon’s signed-rank test was used to define the significance of the difference between groups. For GM3 and GD3 synthase activity in mice experiments, the Student’s t test was used. P < 0.05 was considered statistically significant.

RESULTS

AGEs inhibit pericyte and mesangial cell proliferation. To compare the effects of AGE on pericyte and mesangial cell proliferation, cells were treated with either BSA or 3 μmol/l AGE for 4–7 days. Cell counting revealed that AGEs reduced pericyte and mesangial cell numbers by 33 and 40%, respectively (Fig. 1). Total proteins were also measured, found diminished, and correlated to cell number. The results show similar adverse effects of AGE on both pericyte and mesangial cell proliferation, and led us to investigate common mechanisms involved in the AGE response in these two cell types.

AGEs increase the a-series gangliosides in pericytes and mesangial cells. Previous results from our laboratory suggested that AGEs modulate ganglioside profiles of retinal microvascular cells (17). To this end, ganglioside patterns were analyzed in BRPs and RMCs in response to BSA control or AGEs. As shown in Fig. 2A, ganglioside profile is cell type specific. Under control conditions, the main gangliosides in pericytes were GM3 (63% of all gangliosides detected) and GMI (9%) of the a-series gangliosides (Fig. 2B), and GD3 (28%) of the b-series gangliosides. The mesangial cell profile differed by the very low amount of GD3 (5%) and, in the a-series, by the presence of GD1a (20%), with GM3 remaining the major ganglioside (75%).

An increase of the a-series gangliosides and a decrease of the b-series gangliosides were observed in both AGE-treated cell types. In pericytes, the a-series gangliosides...
GM3 and GM1 were increased by 40%, whereas the b-series ganglioside GD3 was decreased by 24% (Fig. 3A). In mesangial cells, GM3 was increased by 33%, whereas GD3 was decreased by 30%; GD1a levels were not affected (Fig. 3B). Similar results were obtained by autoradiography analysis after galactose labeling (data not shown). As the a-series GM2 in BRPs, GM2 and GM1 in RMCs were hardly detectable by resorcinol staining, cells were labeled with D-[U-14C]galactose at high specific activity, and gangliosides were then analyzed in control and AGE-treated cells. Results revealed that GM2 was also increased by 55% in BRPs (Fig. 3C) and that GM2 and GM1 were increased by 25–35% in RMCs (Fig. 3D). These results indicate that AGEs induce similar ganglioside pattern modifications in both pericytes and mesangial cells. They also suggest that the increase of the a-series gangliosides might be a common mechanism underlying the decrease of cell proliferation.

**AGEs modulate GM3 and GD3 synthase activities in pericytes and mesangial cells.** To investigate the mechanism responsible for the observed a-series ganglioside increase, GM3 and GD3 synthase activities were measured in control and treated cells. Results presented in Fig. 4A show that AGE treatment increased GM3 synthase activity by ~80% in pericytes and 50% in mesangial cells, most likely through an increase of Vmax of the reaction (data not shown). In parallel, AGE treatment decreased GD3 synthase activity by ~60% in both cell types (Fig. 4B). These results suggest that AGEs exert similar mechanisms in RMCs and BRPs leading to a-series ganglioside accumulation.

**a-Series gangliosides inhibit pericyte and mesangial cell proliferation.** Previous literature data have described gangliosides as growth suppressors, notably in mesangial cells (18), and implicated the a-series gangliosides in inhibition of cell proliferation (18–23). Thus, we investigated whether exogenous addition of the a-series gangliosides affects pericyte and mesangial cell proliferation. Cells were treated during one passage with 50 μmol/l gangliosides and, as a control, with the nonsialylated precursors glucosylceramide and lactosylceramide. Figure 5A shows that GM2, GM1, and GD1a inhibited most
efficiently pericyte and mesangial cell proliferation by 15–30%. GM3 slightly inhibited pericyte proliferation but had no significant effect on mesangial cells. Glucosylceramide and lactosylceramide used as controls had no effect. Because GM2 and GM1 are increased in BRPs and RMCs in response to AGEs, and because they alter proliferation of both cell types, we hypothesized that these a-series gangliosides might be common mediators of the AGE effect. Thus, cells were treated with AGEs in the presence of anti-GM1 and anti-GM2 antibodies, and proliferation was measured. In the control BSA-treated cells, proliferation was not different in the presence or absence of anti-ganglioside antibodies (data not shown), and addition of IgG as a control to the AGE treatment did not affect the AGE response. Treatment of cells with AGEs in the presence of anti-GM2 and anti-GM1 antibodies partially prevented the proliferation decrease of pericytes and mesangial cells (Fig. 5B). Together, these observations suggest that the a-series gangliosides, and in particular GM2 and GM1, are common mediators of the AGE-induced inhibition of proliferation of pericytes and mesangial cells.

**GM3 synthase SiRNA partially protects against the AGE effects.** To further support the role of a-series gangliosides in mediating the AGE effects, RMCs were transfected with GM3 synthase SiRNA. Preliminary experiments showed that the designed SiRNA indeed inhibited GM3 synthase activity in RMCs (Fig. 6A). Next, proliferation was measured in the transfected and treated cells. As shown in Fig. 6B, the effects of AGEs on RMC proliferation were partially reversed in cells transfected with GM3 synthase SiRNA. These results clearly show the implication of gangliosides in mediating the AGE effects. It must be indicated that the partial effects could be explained by the following: 1) the AGE effects were less potent than in previous experiments because cells were treated at higher confluence to improve transfection efficiency, and 2) GM3 synthase activity was inhibited only by ~50%. In fact, SiRNA concentrations >400 nmol/l were not used to avoid nonspecific and toxic effects.

**GM3 and GD3 synthase activities and GM3 levels are modified in the renal cortex of diabetic mice.** To assess the effect of a diabetic environment on ganglioside biosynthetic pathway ex vivo, GM3 and GD3 synthase activities were measured on homogenates of renal cortex of the diabetic db/db mouse model. Results presented in Fig. 7A and B show that GM3 synthase activity was increased by 80%, whereas GD3 synthase activity was decreased by 50% in diabetic mice renal cortex compared with controls (db/m). Gangliosides were also analyzed and revealed an increase, although not statistically significant, of GM3 levels in the renal cortex of diabetic db/db mice (Fig. 7C). Altogether, these results support the pathophysiological significance of the results obtained in RMCs and BRPs treated with AGEs.

**DISCUSSION**
Numerous studies have pointed out the crucial role of pericytes and mesangial cells in the development of diabetic retinopathy and diabetic nephropathy, respectively. Indeed, retinal pericyte loss is one of the earliest hallmarks of diabetic retinopathy, and growth arrest, hyper-

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**FIG. 5. a-Series gangliosides inhibit cell proliferation.** A: Pericytes or mesangial cells were treated with ganglioside-BSA complexes for one passage. B: Cells were treated with 3 μmol/l BSA or AGEs in the presence or absence of 5 μg per well of IgG, anti-GM2, or anti-GM1 polyclonal antibodies. At the end of the treatment, total proteins were measured. Results are expressed as a percentage of the control and represent means ± SE of five to six independent experiments, each performed in triplicates. *P < 0.05 vs. control (A) or AGE-treated cells (B). Gluc, glucosylceramide; Lac, lactosylceramide.
trophy, and loss of renal mesangial cells are typical features of diabetic nephropathy. Thus, regulation of these two cell type proliferations appears to be a pivotal pathophysiological process in microvascular complications. AGEs, glycated protein derivatives that notably accumulate in the kidneys (24) and retina (25) of diabetic patients, have been widely involved in the development of microvascular complications (5). In the present study, we investigated the effect of AGEs on BRP and RMC proliferation. Results showed that AGE treatment indeed inhibited both cell type proliferations. These findings are consistent with previous results obtained with other types of AGEs in pericytes (26,27) and mesangial cells (28,29), thus highlighting their pathophysiological relevance.

The effects of AGEs have been extensively studied. However, mechanisms leading to alteration of cell function and viability remain incompletely understood. On the other hand, although gangliosides play a major role in cell growth and apoptosis, their potential involvement in diabetic microvascular complications has been poorly studied. In the present work, we show that treatment of BRPs and RMCs with AGEs resulted in an increase of the a-series gangliosides concomitant with a decrease of the b-series in both cell types. This increase could be explained by the observed concomitant activation of GM3 synthase and inhibition of GD3 synthase activities, suggesting that AGEs regulate activity of these enzymes through yet undefined mechanisms.

On the other hand, evidence that the a-series gangliosides are likely to be responsible for the observed proliferation decrease has been provided in the present study. Exogenous a-series ganglioside application, particularly GM2 and GM1, inhibited BRP and RMC proliferation. Moreover, treatment of cells with AGEs in the presence of anti-GM1 or anti-GM2 partially protected BRPs and RMCs against the proliferation decrease caused by AGEs. Finally, inhibition of GM3 synthase using specific siRNA partially reversed the AGE effects on RMC proliferation. Together, these results suggest that the a-series gangliosides, and in particular GM2 and GM1, are involved in mediating the anti-proliferative effect of AGEs on BRPs and RMCs. The partial protection observed using anti-GM2 and anti-GM1 antibodies or siRNA approach might suggest that other pathways are also involved in mediating the AGE effect. For example, a cascade involving oxidative stress and ceramide accumulation has been proposed to induce BRP apoptosis in response to AGEs (16). The role of the a-series gangliosides is further supported by numerous studies showing that a-series gangliosides decrease cell proliferation by modulating growth factor receptor signaling. For example, a-series gangliosides (GM3, GM2, GM1, GD1a) have been described to inhibit epidermal growth factor receptor activity and platelet-derived growth factor receptor phosphorylation and signaling in different cell types (19–22,30–32).

The potential role of gangliosides in diabetic microvascular complications is also supported by ex vivo observations. Indeed, the present results show that GM3 and GD3 synthase activities are increased and decreased, respectively, and that GM3 tends to accumulate in the renal cortex of diabetic db/db mice. Moreover, GM3 has been shown to accumulate in the kidney of streptozotocin-induced diabetic rats, and kidney hypertrophy was inhibited when ganglioside biosynthesis was blocked, suggesting a crucial role of gangliosides in diabetes-induced renal hypertrophy (33).

On the other hand, recent studies have suggested the involvement of GM3 synthase and a-series gangliosides in insulin resistance. Thus, GM3 synthase activity and its mRNA have been shown to be increased in adipocytes treated with low concentrations of tumor necrosis factor-α, and GM3 synthase mRNA levels were found to be increased in adipose tissues of Zucker fa/fa rats (34). Furthermore, GM3 synthase knockout mice showed increased sensitivity to insulin by enhanced insulin receptor phosphorylation, and mice were protected against high-fat diet–induced insulin resistance (35). Finally, mice overexpressing the human sialidase NEU3 showed reduced insulin-stimulated phosphorylation of the insulin receptor and the insulin receptor substrate, suggesting that GM2 and GM1, the possible sialidase products in transgenic tissues, attenuate insulin signaling (36).

In conclusion, our data suggest that the a-series gangliosides, and in particular GM2 and GM1, are mediators of the

FIG. 6. Transfection with GM3 synthase siRNA protects RMCs. A: Thin-layer chromatography separation of GM3 product obtained from GM3 synthase activity in homogenates of RMCs. Mesangial cells were transfected with increasing concentrations of GM3 synthase siRNA, and GM3 synthase activity was measured after 72 h. Ctr, cells transfected with BSA and represent means ± SE of six independent experiments. *P < 0.05 vs. AGE-treated control cells.

B: Twenty-four hours after transfection with 400 μmol/l GM3 synthase siRNA, cells were treated with 3 μmol/l BSA control or AGEs. At the end of treatment, total proteins were measured. Results are expressed as the percentage of control BSA and represent means ± SE of six independent experiments. *P < 0.06 vs. AGE-treated control cells.
adverse AGE effects on pericyte and mesangial cell proliferation. Further, the ganglioside pathway appears to be a new and common AGE mechanism of action in both BRPs and RMCs. These data also raise GM3 synthase and α-series gangliosides as potential targets for the treatment of both diabetic retinopathy and diabetic nephropathy.

REFERENCES


FIG. 7. GM3 and GD3 synthase activities, and GM3 levels are modulated in renal cortex of diabetic mice. GM3 (A) and GD3 (B) synthase activities were measured on homogenates of renal cortex from control (db/m) and db/db mice. Control activities were 0.6 and 21 pmol·h⁻¹·mg⁻¹ proteins for GM3 and GD3 synthase, respectively. C: GM3 levels of control (db/m) and db/db mice renal cortex are represented. In control mice, GM3 levels were 66 ± 9 ng/mg proteins. Results are expressed as a percentage of the control and represent means ± SE of four to six animals. *P < 0.05 vs. control mice.


