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Glycosphingolipid Changes Induced by Advanced Glycation End-Products

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The effects of advanced glycation end-products (AGEs) on retinal microvascular cell glycosphingolipids were investigated as a potential pathogenic mechanism of diabetic retinopathy. The results obtained showed that, in microvascular retinal endothelial cells and pericytes, AGEs increased the amount of all glycosphingolipids studied (from 25 to 115% depending on the glycosphingolipid species), except for a specific ganglioside, GD3, which decreased by 35% only in pericytes. Glycosphingolipid profiles and GM3 fatty acid analysis did not show any qualitative differences after incubation with AGEs, suggesting that AGEs only induced quantitative changes in cell glycosphingolipids. These results show a new metabolic effect of AGEs, which could be involved in the microvascular alterations observed in diabetic retinopathy.

Key Words: **diabetic retinopathy; glycosphingolipids; advanced glycation end products; endothelial cells; pericytes; gangliosides.**

Diabetic retinopathy is one of the most common cause of blindness in developed countries. This complication of diabetes mellitus is characterized by alterations of retinal microvessels, leading to early morphological changes such as thickening of the capillary basement membrane and loss of pericytes. Although pathogenic mechanisms involved still remain poorly understood, modification of cell–cell and cell–matrix interactions is believed to play an important role (1).

Abbreviations used: AGEs, advanced glycation end products; BREC, bovine retinal endothelial cells; BRP, bovine retinal pericytes; BSA, bovine serum albumin; C, chloroform; CMH, ceramide monohexosides; CDH, ceramide dihexosides; CTH, ceramide trihexosides; HPTLC, high performance thin-layer chromatography; M, methanol; PBS, phosphate-buffered saline.

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One possible mechanism contributing to diabetic retinopathy is the increased covalent and irreversible modification of proteins by reducing sugars, due to hyperglycemia. This nonenzymatic glycosylation of intra and extracellular proteins leads to the enhanced formation and accumulation of advanced glycation end-products (AGEs). Several studies have shown that AGEs are involved in the etiopathology of diabetic microangiopathic complications (2). Previous studies have also shown that AGEs could alter the enzymatic glycosylation of glycoproteins in retinal microvascular cells (3).

As observed for glycoproteins, glycosphingolipids are ubiquitous components of the plasma membrane. They are composed of an extracellular hydrophilic oligosaccharide portion and a hydrophobic ceramide portion, embedded in the plasma membrane. Glycosphingolipids seem to play major roles in cell–cell recognition, cell–matrix interactions and cell proliferation (4, 5). In the pathogenesis of diabetic retinopathy, several cellular interactions are believed to be altered, such as interactions between microvascular and blood cells, the basement membrane and the neuronal retina or interactions between the microvascular cells themselves (1). It has been demonstrated that glycosphingolipids are implicated in cell–cell and cell–matrix interactions through different mechanisms (4) such as recognition by endogenous lectins, modulation of adhesion receptors or interaction with other glycosphingolipids. It has also been shown that specific exogenous gangliosides (for example GM3), which are glycosphingolipids containing sialic acid, can modulate the activity of different growth factor receptors (6–8) which have been implicated in the neovascularization process of proliferative diabetic retinopathy (9) and in the modulation of the proliferation of endothelial cells and pericytes (10). Moreover, some specific gangliosides have recently been involved in apoptosis (11, 12), a phenomena that may occur in diabetic retinopathy and lead to the early disappearance of pericytes in the microvessels of the retina (13).

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Because of the importance of glycosphingolipids in cellular interactions, cell growth and apoptosis (processes which are altered in diabetic retinopathy) and the role of AGEs in the pathogenesis of diabetic microangiopathy, we investigated in this study a possible new metabolic effect of AGEs on glycosphingolipids of retinal microvascular cells.

MATERIALS AND METHODS

Materials. Chemicals were obtained from Sigma-Aldrich (St Quentin-Fallavier, France) or Merck (Darmstadt, Germany) unless otherwise specified. ^{14}C-galactose was purchased from DuPont-NEN (Les Ulis, France). C_{18} silica gel columns were from Waters Corporation (Sep-Pak Cartridges, MA). Columns of G25-Sephadex were from Amersham Pharmacia Biotech (Sweden). Neutral glycosphingolipid standards were obtained from Matreya (Pleasant Gap, PA). Ganglioside standards were a kind gift from J. Portoukalian (Faculty of Medicine Lyon-Sud, Oullins, France).

Culture of bovine retinal microvascular cells. Endothelial cells (BREC) and pericytes (BRP) were obtained from bovine retinal microvessels and cultured as previously described (14). Purity of cell cultures was assessed to be more than 90% for BREC and 100% for BRP as described previously (14). To study the effect of advanced glycation end-products, 3 μM (final concentration) of either AGE-BSA or control BSA were added to the cell culture media. AGE-BSA were prepared by incubation of bovine serum albumin (7.2 mg/ml, final concentration) with methylglyoxal (100 mM, final concentration) at 37°C for 50 h (15). Bovine serum albumin (7.2 mg/ml) in the absence of methylglyoxal was incubated in the same conditions and used as a control preparation (control BSA). AGE-BSA and control BSA were then desalted using G-25 Sephadex columns. BREC and BRP were cultured in the presence of modified media for 8 or 15 days, respectively. For metabolic radiolabeling of cell glycosphingolipids, 0.5 μCi/ml of galactose D-[^{14}C(U)] (292 mCi/mmol) was added to the media sixteen hours before recovering the cells.

Isolation and analysis of glycosphingolipids. BREC and BRP were harvested by trypsination and cell pellet washed twice by Dulbecco's phosphate buffered saline. About 10^6 cells were used for each analysis. Extraction and isolation of neutral glycosphingolipids and gangliosides were achieved according to Bouchon *et al.* (16) with minor modifications. Cell pellet was dispersed in 2 ml of chloroform (C)/methanol (M) (1:1, v/v), mixed thoroughly and extracted overnight at 4°C. After centrifugation, the residue was extracted twice with 1 ml of the same solvent and dissolved in 10% sodium dodecyl sulfate for protein determination. The pooled extracts were evaporated to dryness and submitted to a partition with C/M/PBS 1 mM (10:10:7, v/v/v).

The lower phase, containing neutral glycosphingolipids, was analyzed by high performance thin-layer chromatography (HPTLC) on silica gel 60 plates (Merck, Darmstadt, Germany). Glycosphingolipids were loaded on HPTLC plates with an automatic TLC sampler from Camag (Muttenz, Switzerland). The plates were developed first in C/acetone (96:4, v/v) and then in C/M/H_2O (65:25:4, v/v/v) in a horizontal developing chamber from Camag. Neutral glycosphingolipids were visualized by autoradiography and orcinol-H_2SO_4 (17).

The upper phase, containing the gangliosides, was desalted by a C_{18} silica gel column chromatography and analyzed by HPTLC. The plates were developed in C/M/0.2% $CaCl_2$ (55:45:10, v/v/v) and gangliosides were visualized by autoradiography and resorcinol-HCl (17). Neutral glycosphingolipid and ganglioside distribution was determined by densitometric scanning (GS-700 densitometer and Molecular Analyst software from Bio-Rad, Ivry sur Seine, France). Results were adjusted to the recovered protein amount in each sample. Proteins were determined by the method of Schaffner and Weissman (18).

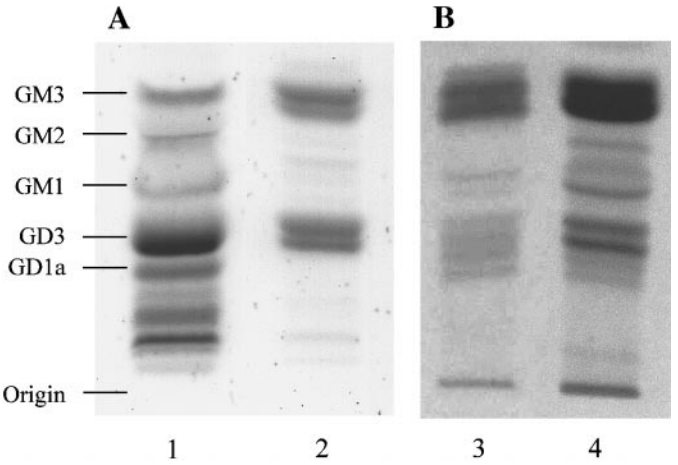

FIG. 1. Radiolabeled gangliosides from bovine retinal microvascular cells. Gangliosides of bovine retinal endothelial cells (BREC) and pericytes (BRP) were metabolically radiolabeled by ^{14}C-galactose, extracted, analyzed by HPTLC and the plates autoradiographed as described under Materials and Methods. (A) Resorcinol stain (B) autoradiogram. Lane 1, reference gangliosides from bovine retina; lane 2, reference gangliosides from human melanoma; lane 3, gangliosides from BREC; lane 4, gangliosides from BRP.

GM3 fatty acid analysis. Gangliosides of BRP and BREC were isolated as described above and visualized on HPTLC plate with primulin. Bands corresponding to GM3 were scraped. Blank silica was also scraped, analyzed as GM3 and used as a control. GM3 was extracted from the silica by elution on small columns with C/M (1:1), C/M (1:2) and methanol. Free fatty acids of GM3 were obtained according to Aveldano and Horrocks (19) after incubation with 0.5 M HCl in acetonitrile/H_2O (9:1, v/v) at 75°C for 2 h. FFA were derivatized as already described using pentafluorobenzene (PFB) and *N*,O-bis(trimethylsilyl)trifluoroacetamide (BSTFA) (20). Free fatty acid derivatives were analyzed by GC-MS as already described (21). Detection was made by negative ion chemical ionization (NICI) in scan mode, with methane as reactant gas.

Statistical data analysis. Data are represented in percentage of control as means ± SEM of independent experiments. To compare two groups (AGE-BSA/control BSA), the Wilcoxon signed-ranks test was used. $P < 0.05$ was considered as statistically significant.

RESULTS

Glycosphingolipid composition of bovine retinal endothelial cells. Analysis by HPTLC and autoradiography of metabolically radiolabeled gangliosides in BREC showed the presence of four double bands comigrating with GM3, GM1, GD3, and GD1a (Fig. 1). GM3 was the main ganglioside (81% ± 2). Bands migrating with GM1, GD3, and GD1a represented 3% ± 1, 6% ± 1 and 9% ± 2 ($n = 7$) of the total gangliosides, respectively. Concerning the neutral glycosphingolipids, bands comigrating with ceramide monohexosides (CMH), ceramide dihexosides (CDH), ceramide trihexosides (CTH), and globoside were identified, representing 48% ± 2.5, 4% ± 1, 6% ± 2 and 30% ± 4, respectively (Fig. 2). One unidentified band migrated between CMH and CDH and represented 10% of the total neutral glycosphingolipids.

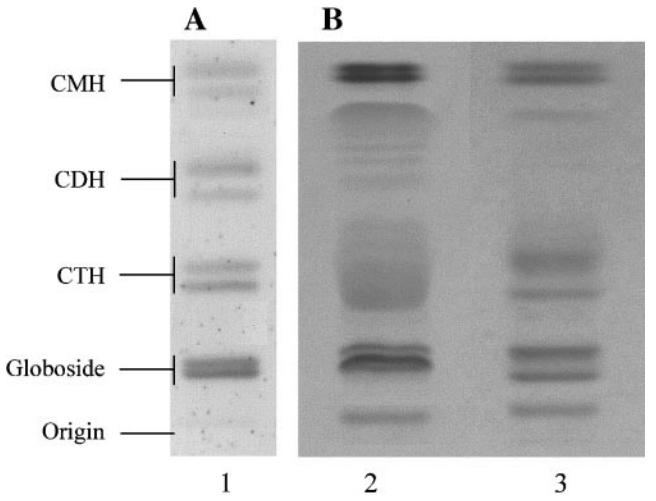

FIG. 2. Radiolabeled neutral glycosphingolipids of bovine retinal microvascular cells. Neutral glycosphingolipids of bovine retinal endothelial cells (BREC) and pericytes (BRP) were metabolically radiolabeled by ^{14}C-galactose, extracted, analyzed by HPTLC and the plates autoradiographed as described under Materials and Methods. (A) Orcinol stain (B) autoradiogram. Lane 1, standards neutral glycosphinogolipid mixture; lane 2, neutral glycosphingolipids from BREC; lane 3, neutral glycosphingolipids from BRP.

Glycosphingolipid composition of bovine retinal pericytes. Metabolically radiolabeled gangliosides in BRP showed the presence of four double bands comigrating with GM3, GM1, GD3, and GD1a, and one band migrating near GM2 (Fig. 1). GM3 and GD3 were the main gangliosides present in BRP. They represented 71% ± 6 and 17% ± 4 (n = 9) of the total BRP gangliosides, respectively. The bands migrating with GM1, GD1a, and GM2 represented 8% ± 1.6, 1.2% ± 0.8 and 2.5% ± 1 of the total gangliosides, respectively. The same analysis for the metabolically radiolabeled neutral glycosphingolipids showed the presence of three double bands comigrating with CMH, CTH and globoside and one unidentified band migrating between CMH and CDH (Fig. 2). Globosides, CTH and CMH represented 26% ± 6, 35% ± 4 and 34% ± 4 of the total neutral glycosphingolipids, respectively.

Effects of advanced glycation end products on glycosphingolipids of bovine retinal cells. After incubation of the cells (BREC and BRP) with 3 μM of advanced glycation end-products (AGE-BSA) or control, the pattern of the metabolically radiolabeled gangliosides and neutral glycosphingolipids was the same as the control cells. However, a significant increase in the content of all metabolically radiolabeled glycosphingolipids was observed in both cell types, except the ganglioside GD3, which was decreased only in BRP (Fig. 3). For gangliosides, increases ranged from 35% (GM3) to 114% (GM1) in BREC, and from 31% (GM3) to 59% (GM1) in BRP. In BRP, GD3 was decreased by 35%. Concerning neutral glycosphingolipids, increases observed ranged from 24% to 47% in BREC and from 22% to 80% in BRP, depending on the neutral glycosphingolipid species. Gangliosides of BRP were also analyzed by HPTLC and resorcinol-HCl staining in order to measure sialic acid content. Densitometric scanning showed almost the same variations that analysis after metabolic radiolabeling of gangliosides. Indeed, analysis of the resorcinol stained-plates showed an augmentation of 21% for GM3 and a decrease of 32% for GD3 in BRP incubated with AGE-BSA compared to control BSA (data not shown).

GM3 fatty acids analysis. GM3 fatty acids of BREC and BRP were analyzed by GC-MS. The major fatty acids in both cell types were palmitic acid (16:0, 38%), stearic acid (18:0, 24%), oleic acid (18:1, 14%), myristic acid (14:0, 7%), and palmitoleic acid (16:1, 5%). Other minor fatty acids such as 20:0, 20:1, 22:0, 24:0, 24:1 represented each 1% to 3% of the total fatty acids. Monohydroxylated fatty acids (16:0-OH and 18:0-OH) were present in a very small proportion (0.6%). No significant differences (n = 3) were observed between GM3 fatty acid composition of BREC and BRP incubated in presence of control BSA or AGE-BSA.

DISCUSSION

The results obtained in this study show for the first time that advanced glycation end-products, which are

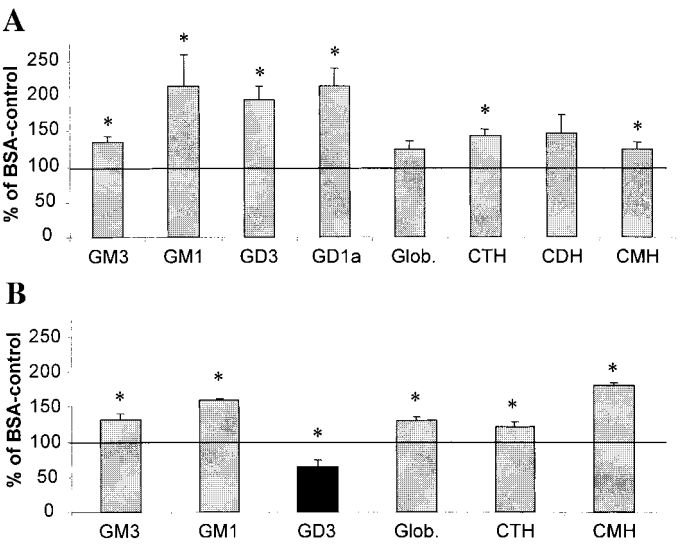

FIG. 3. Effects of advanced glycation-end products on radiolabeled glycosphingolipids of retinal microvascular cells. Endothelial cells (A) and pericytes (B) from bovine retinal microvessels were incubated in presence of advanced glycation end-products (AGE-BSA) or albumin (BSA-control). Gangliosides and neutral glycosphingolipids were metabolically radiolabeled by ^{14}C-galactose, extracted and isolated as described under Materials and Methods. They were analyzed by HPTLC and autoradiography and quantified by densitometric scanning. Results were adjusted to the protein quantity and expressed in percentage of control. The percentages are represented as means ± SEM values of five independent experiments. *$P < 0.05$ vs "control." Glob., globoside.

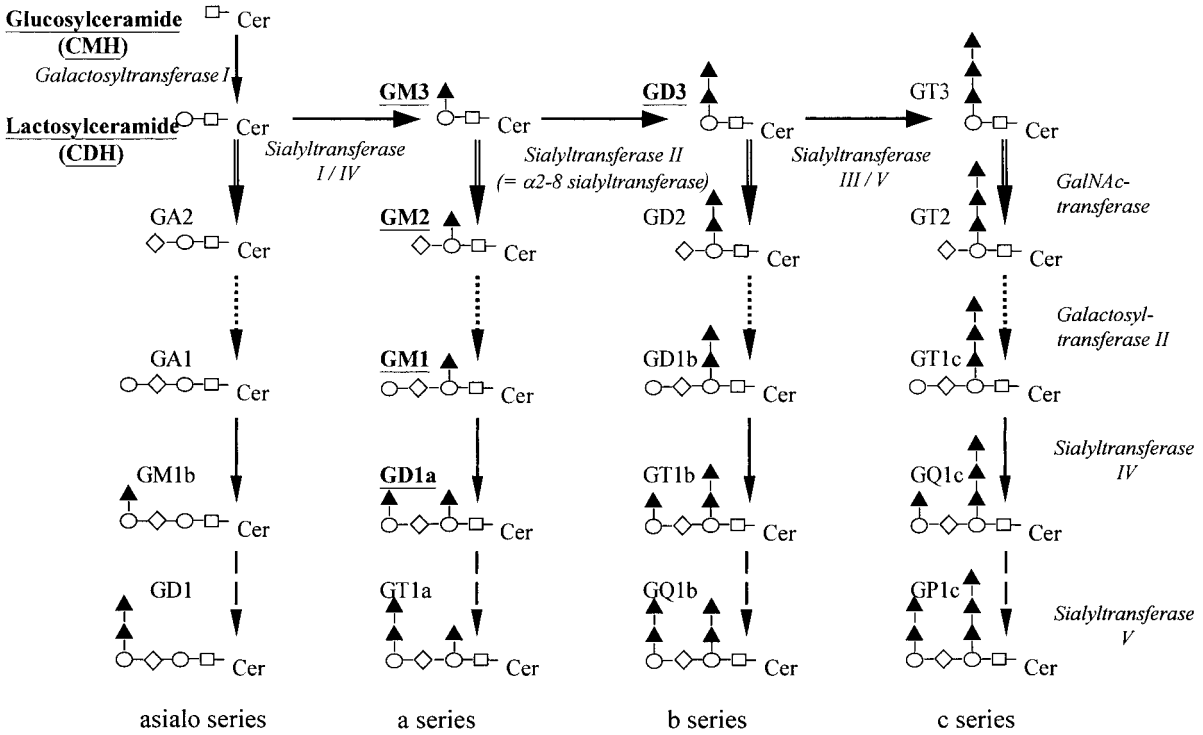

FIG. 4. Pathways of ganglioside biosynthesis. □, glucose; ○, galactose; ◇, *N*-acetylgalactosamine; ▲, *N*-acetylneuraminic acid. Bold and underlined glycosphingolipids are those detected in bovine retinal microvascular cells.

believed to play an important role in diabetic retinopathy, can induce modifications in the composition of glycosphingolipids in retinal microvascular cells such as pericytes and endothelial cells.

Although glycosphingolipids play a major role in cellular interactions, cell growth and apoptosis, processes which are altered in diabetic retinopathy, they have never been studied in retinal microvascular cells. Therefore, before investigating the effects of AGEs on glycosphingolipids of BREC and BRP, determination of the glycosphingolipid composition of these cells was performed. For this purpose, glycosphingolipids of BREC and BRP were metabolically radiolabeled by ^{14}C-galactose and analyzed by HPTLC and autoradiography. This sensitive methodology was chosen because of the small amount of glycosphingolipids present in vascular cells and the difficulty to grow primary cultures of BRP and BREC in large quantity. Glycosphingolipids, which all migrated in doublets, were identified by their comigration with standards on the HPTLC plates. We found that the major ganglioside of BRP and BREC was GM3 (71 and 81% of the total gangliosides, respectively), which agrees with the ganglioside composition of other vascular cells such as human umbilical vein endothelial cells and smooth muscle cells (22) or bovine aortic endothelial cells (23), where GM3 is the predominant ganglioside.

After incubation of the cells with AGE-BSA, all radiolabeled glycosphingolipids were increased in the two cell types, except ganglioside GD3 which was decreased in BRP. Quantification of gangliosides by measuring their sialic acid content with resorcinol staining showed the same glycosphingolipid modifications observed by metabolic radiolabeling, suggesting that AGEs induced changes in the amount but not in the turnover rate of glycosphingolipids. GM3 fatty acid analysis showed that AGEs did not induce any modifications in the fatty acid pattern of GM3 from BREC and BRP. These results suggest that AGEs induced quantitative, but not qualitative, changes in glycosphingolipids of these cells.

As previously discussed, glycosphingolipids were increased by incubation with AGEs (except for GD3 in pericytes). Because of the sequential biosynthesis of glycosphingolipids (Fig. 4), this global increase could be explained by an augmentation of biosynthetic precursors, such as ceramides or glucosylceramides (CMH). Nevertheless, the increase in GM1, GD3, and GD1a in BREC was significantly higher than that in GM3, the common precursor of these gangliosides. This suggests that mechanisms involved in AGEs effects on cell glycosphingolipids could be more complex than a simple increase of precursor metabolites. In BRP, a specific ganglioside, GD3, was unexpectedly decreased by AGEs, indicating a very specific effect of AGEs on the metabolism of this particular ganglioside. Its decrease cannot be explained by its transformation into GD2 or GT3 since these products are not detected in

cells incubated with AGEs. The GD3 diminution could be explained by an inhibition of the enzyme responsible of its synthesis (the α-2,8 sialyltransferase, cf. Fig. 4) by AGEs and/or an activation of a sialidase responsible of its degradation. This specific decrease of GD3, whereas all other glycosphingolipids increase concomitantly, merits a particular attention and needs further investigations to better understand how AGEs can alter the metabolism of this particular ganglioside and what its physiological consequences are. Unlike BRP, GD3 from BREC was increased similarly to the other gangliosides in response to AGEs. The fact that AGEs induced two different responses in the two cell types is not surprising. Several studies have already reported different biological effects of AGEs on BRP and BREC (3, 14, 24) and it is known that microvascular pericytes and endothelial cells are differently affected by the diabetic environment during the evolution of retinopathy (25).

The glycosphingolipid modifications observed might be involved in the pathogenic mechanisms leading to retinal microvascular abnormalities present in diabetic retinopathy. Pericyte loss and endothelial cell proliferation are hallmarks of diabetic retinopathy. *In vitro,* it has been shown that AGEs decrease retinal pericyte proliferation and enhance retinal endothelial cell growth (26). The glycosphingolipid changes observed in these cells could be involved in this modulation of cell proliferation by AGEs. Indeed, several studies have shown that glycosphingolipids play a major role in cell growth. Exogenous gangliosides, which are incorporated in the cell membrane, are known to alter or enhance cell proliferation, depending on the ganglioside and the cell type (27, 28). More recently, it has been found that changes in cell glycosphingolipid content correlate with alteration of cell growth (29) and modulation of epidermal growth factor (EGF) receptor autophosphorylation (30). Other growth factor receptors, such as the platelet-derived growth factor (PDGF) receptor (8) or the fibroblast growth factor (FGF) receptor (7), are also modulated by gangliosides. Some of these growth factor receptors are present in pericytes and endothelial cells and have been involved in the pathogenesis of diabetic retinopathy. These findings allow us to speculate about a possible role of glycosphingolipid composition in the modulation of cell proliferation by AGEs. Apoptosis seems to be an important mode of pericyte drop out in human diabetic retinopathy (13). It is known that gangliosides such as GM1 or GD3 are involved in apoptotic processes (11, 12). Therefore, it could also be possible that ganglioside changes induced by AGEs participate to apoptosis in diabetic retinopathy.

The mechanisms involved in the biological effects of AGEs, such as cell growth modulation or apoptosis, remain largely unknown. Most of the AGE effects have been suggested to be mediated by the interaction of AGEs with specific membrane receptors and induction of oxidative stress inside the cell (2). Experiments are in progress to know whether oxidative stress could be implicated in the modification of glycosphingolipid composition induced by AGEs in BREC and BRP. However, other regulation mechanisms at the translational and post-translational level cannot be excluded since enzymatic glycosylation is a complex and sophisticated metabolic pathway of which the regulation mechanisms are not well known.

In conclusion, this study shows a new metabolic action of advanced glycation end-products, whereby specific steps in biosynthetic glycosphingolipid pathway are perturbed. These alterations could be involved in the cellular dysfunctions observed in retinal microvessels during diabetic retinopathy. Nevertheless further experiments will be needed to determine the physiological relevance of these glycosphingolipid changes.

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