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Exogenous advanced glycosylation end products induce diabetes-like vascular dysfunction in normal rats: a factor in diabetic retinopathy

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Abstract *Background:* Diabetic retinopathy has been shown to be directly associated with the degree and duration of hyperglycemia, and advanced glycosylation end products (AGEs) have been implicated in this pathological process. The purpose of the experiments reported here was to study the effect of AGE deposition on retinal vascular damage which leads to diabetic retinopathy. *Methods:* Intravenous injection of exogenous AGEs was used to treat wild-type non-diabetic Sprague-Dawley rats. One of the two retinal slides from each animal was treated using immunohistochemical staining to label retinal vascular AGE deposition, the other H&E staining for counting of capillary pericytes. The results were compared with the findings in untreated wild-type and diabetic controls and in rats treated with unmodified rat serum albumin (RSA). *Results:* After 2 weeks of continuous treatment, AGEs were identified in the retinal vascular tissue of the AGE-RSA-injected group. The average number of retinal capillary pericytes per 10×100 microscope power field was 4.313±0.34 (mean ± SD) in the AGE-RSA-injected group, compared with 5.798±0.481 in the control group ($P<0.01$). *Conclusion:* These experiments demonstrate that AGEs, independent of other metabolic factors, can induce vascular change resembling that of diabetic retinopathy.

Introduction

Along with the increasing prevalence of diabetes mellitus, diabetic retinopathy is becoming one of the leading causes of visual impairment or blindness, accounting for 13% of all cases of visual loss [9]. The risk of retinopathy is directly related to the degree and duration of hyperglycemia [6]. After diabetes mellitus has been present for 20 years, almost all persons in whom the onset of diabetes occurred before the age of 30 years have some evidence of retinopathy. The 20-year prevalence of any type of retinopathy is about 80% among older diabetics who require insulin and 20% among those who do not require insulin [2]. Early detection of retinopathy is difficult, yet on the other hand, end-stage retinopathy is very difficult to treat [12]

The mechanism of the development of diabetic retinopathy is not entirely clear. It has been shown that in patients with poorly controlled diabetes, advanced glycosylation end products (AGEs) can increase abnormally and become deposited in renal vascular tissues, leading to capillary vasculopathy in the kidneys [11]. Toxic effects of AGEs result from structural and functional alterations in plasma and extracellular matrix (ECM) proteins, in particular from cross-linking of proteins and interaction of AGEs with their receptors and/or binding proteins. It has been reported that in mesangial and endothelial cells, the interaction between AGEs and their receptors causes enhanced formation of oxygen radicals with subsequent activation of nuclear factor-κB and release of pro-inflammatory cytokines (interleukin-6, tumor necrosis factor-α), growth factors (transforming growth factor-β1, insulin-like growth factor-1), and adhesion molecules [4].

The exact role of AGEs in the development of diabetic retinopathy remains to be elucidated. The study re-

ported here attempted to show how to introduce exogenous AGEs to young, non-diabetic animals and to study the effects of AGE deposition alone on the development of retinal vasculopathy.

Materials and methods

Preparation and characterization of exogenous AGE

Three grams of D-glucose and 1.6 g of rat serum albumin (RSA) were dissolved in 10 ml 0.5 M phosphate buffer, and the mixture was incubated at 37 °C for 90 days. The product was purified through dialysis with 0.15 M NaCl/0.02 M Na_3PO_4 buffer. Protein concentration was determined by the Lowry method, and fluorescence was measured under activating wavelength of 370 nm. SDS-PAGE was used to show difference of molecular weight between that of the purified incubation product and untreated RSA. Immunoprecipitation reaction was also done using multiclonal antibodies against AGEs which were made previously [5].

Treatment of Sprague–Dawley rats

Two-month old Sprague–Dawley rats were randomly assigned to four groups (n=12): wild-type control, diabetes control, RSA injected, and AGE-RSA injected. Type 1 (insulin-dependent) diabetes mellitus was induced chemically in the diabetes control group using the method described previously [5]. The RSA-injected rats received a daily tail-vein injection of RSA 40 mg/kg/day; the AGE-RSA-injected group was treated similarly but with prepared AGE-RSA. Fasting glucose levels were recorded weekly. Care and use of the rats adhered to the principles set forth in the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

After 2 weeks of treatment, four rats from each group were catheterized via the ascending aorta under deep anesthesia and polyformaldehyde was infused for blood displacement. Enucleation was performed after retinal blood vessels changed to lucidification. The enucleated eyes were fixed for 48 h in polyformaldehyde solution; retina was isolated from the anterior segment and vitreous body, treated with 3% trypsin, and made into a slide using the methods of Zhang [15]. One of the two retinal slides from each animal was treated using ABC immunohistochemical staining to label retinal blood vessel AGEs, the other using hematoxylin and eosin (H&E) stain for capillary pericytes and endothelial cells. The immunohistochemistry protocol used multiclonal anti-AGE as primary antibodies, horseradish peroxidase-linked goat anti-rabbit immunoglobulin antibodies as secondary antibodies, and DAG as staining agent [5]. The degree of positive immunochemical staining was evaluated [5], and pericytes were counted using the H&E-stained slides under oil-immersion field (10×100) [13]. The retina was divided into nine zones under the microscope by two pairs of parallel lines perpendicularly intersecting each other and centered around the optic papilla. The number of pericytes in each zone was counted under an oil-immersion field. The result of each experimental animal was the average of these nine values.

The rest of the rats from the AGE-RSA-injected group and the RSA-injected group were deeply anesthetized. Fluorescence fundus angiography (FFA) was performed with intraperitoneal injection of 0.6 ml of 10% sodium fluorescein using the Heidelberg Eye Explorer Image System (Heidelberg, Germany) and images were captured for up to 30 min after injection of the dye. Then all eyes were dissected along the corneoscleral border to collect retina. The retinae were fixed for 4 h and subjected to pathological section.Two random retinal slides from each eye were treated using the Envision immunohistochemical staining method to label Flt-1,a type of vascular endothelial growth factor (VEGF) receptor, using anti-Flt-1 polyclonal antibody (Santa Cruz Corp.). Positive cells were counted in five zones under an oil-immersion field (10×100).Another two retina sample from each group were prepared for ultrathin sections (Ultramicrotome, LKB-1, Sweden) and subsequently photographed by transmission electron microscopy (Jeol JEM-1200EX, Japan).

AGE immunohistochemical staining data were analyzed using the H test to determine statistical significance. Data on numbers of pericytes and Flt-1-positive cells were processed using the F test, and the mean values in the different groups were compared using the Dunnett test. $P<0.05$ was considered significant.

Results

The results of average blood glucose are shown in Table 1. Rats from the diabetic control group showed higher blood glucose, averaging 24.6±1.290 mmol/l, than other groups. AGE-RSA-injected and RSA-injected rats displayed no blood glucose changes.

Preparation and characterization of AGE-RSA

The mixture of D-glucose and RSA turned into a stable, viscous brown AGE-RSA product that yielded fluorescence of 440 nm under activation at 370 nm. The intensity differed significantly from that of original RSA (43.4 U/mg vs 0.37 U/mg). SDS-PAGE electrophoresis showed that AGE-RSA is a glycosylation end product with a greater molecular weight thanRSA (68 kDa) (Fig. 1). Immunoprecipitation experiments also showed that the end product reacted with multiclonal anti-AGE antibodies prepared previously [5].

Table 1 Experimental animals' average fasting glucose level, retinal AGE immunohistochemical staining, and average pericyte numbers

Group (n=4)	Fasting glucose level (mean ± SD, mmol/l)	AGE staining (scale of – to ++++)	Pericyte number per oil immersion field (mean ± SD)
Wild-type control	2.9±0.283	–	6.125±0.189
Diabetic control	24.6±1.290	–	5.979±0.119
RSA injected	2.535±0.233	–	5.798±0.481
AGE-RSA injected	2.75±0.349	++*	4.313±0.340**

* H =15, P<0.01; ** F=7.164, P <0.01

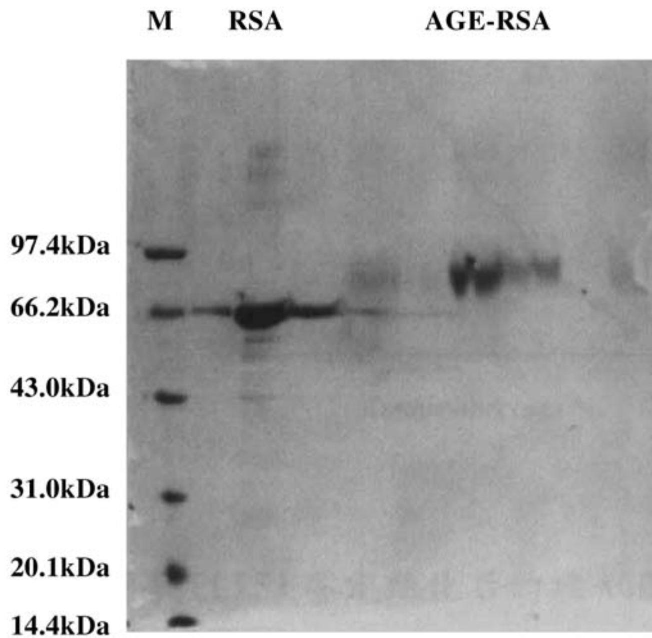

Fig. 1 The result of SDS-PAGE electrophoresis (*M* protein marker, *RSA* (unchanged) 68 kDa, *AGE-RSA* ≈85 kDa)

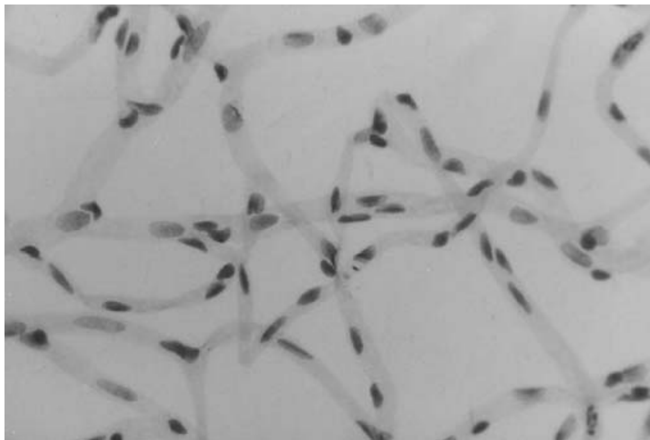

Fig. 2 H&E staining of retinal vascular tissue from the AGE-RSA-injected group (10×40 power field). The AGE-RSA-injected group has decreased pericyte density

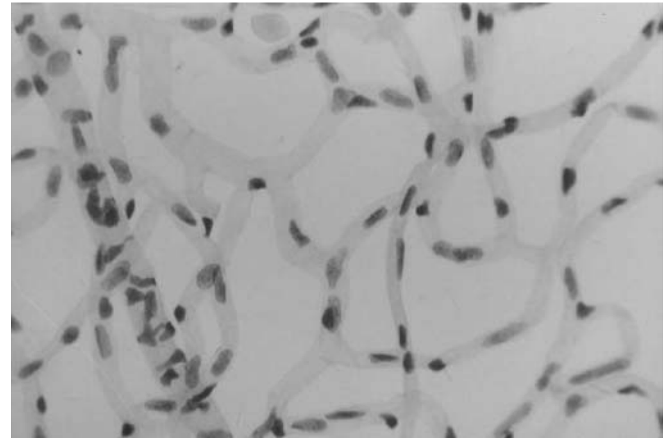

Fig. 3 H&E staining of retinal vascular tissue from the RSA-injected group (10×40 power field). The RSA-injected group shows no statistical difference from the wild-type control group in average pericyte numbers

Morphological observation

The retinal capillary slides obtained from each group of SD rats gave a clear picture of the capillary structure and its histopathological change. Only the AGE-RSA-injected group showed a significant change in both the appearance and the number of pericytes under the same microscopic visual field. (Figs. 2, 3; Table 1). Through analysis of variance, the average numbers of pericytes are statistically different (F=7.164, P<0.01).Neither the short-term (2-week) diabetic control group nor the RSA-injected group differed statistically from the wild-type control group in average number of pericytes. Further comparisons among the different groups are shown in Table 2. The AGE-RSA-injected group had a lower pericyte density than both the wild-type control group and the RSA-injected group, and these differences were statistically significant (Dunnett test: $d'_{(3\cdot 0.01)}$=1.5989, $d'_{(1\cdot 0.01)}$=1.351; P<0.01). Pericyte swelling and change in shape, nuclear hypochromia, mitochondrial swelling, and elevated electronic density of the retinal basement membrane were also noted (Figs. 4, 5), similar to the findings in rats with more than 6 months of diabetes [13]. However, no retinal capillary or choroidal dye leakage was found in the AGE-RSA-injected group or the RSA-injected group (Figs. 6, 7).

Table 2 Comparisons of average pericyte densities in the experimental groups (Dunnett test)

Comparison	Δ	S_{Δ}	d'	P
Diabetic control vs wild-type control	0.145	0.44291	$d'_{(1,0.05)}$=0.966	P>0.05
RSA injected vs wild-type control	0.3275	0.44291	$d'_{(2,0.05)}$=1.107	P>0.05
AGE-RSA injected vs wild-type control	1.8125	0.44291	$d'_{(3,0.01)}$=1.5989	P<0.01
AGE-RSA injected vs RSA injected	1.485	0.44291	$d'_{(1,0.01)}$=1.351	P<0.01

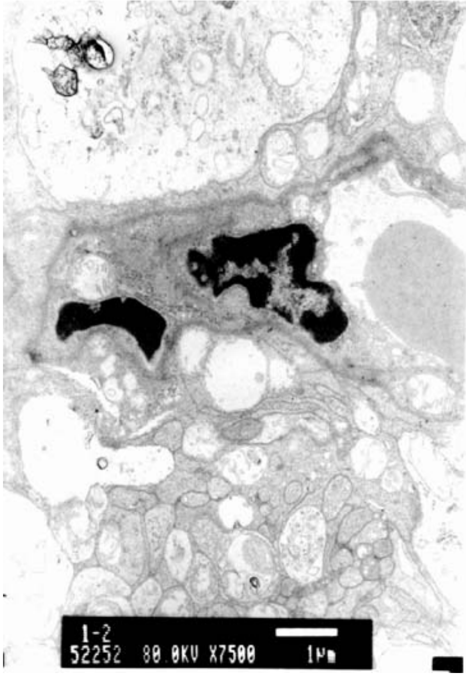

Fig. 4 Transmission electron micrograph of retinal vessels from the AGE-RSA group (*bar* 1 μm)

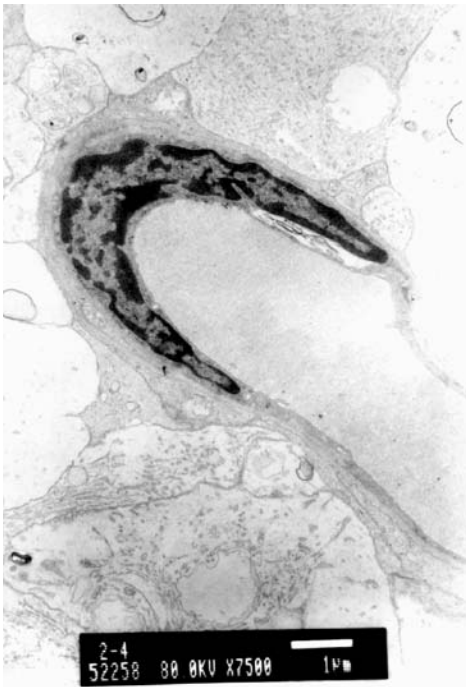

Fig. 5 Transmission electron micrograph of retina vessel from the RSA group (*bar* 1 μm). There are no significant morphological abnormalities in the samples from the RSA-injected group

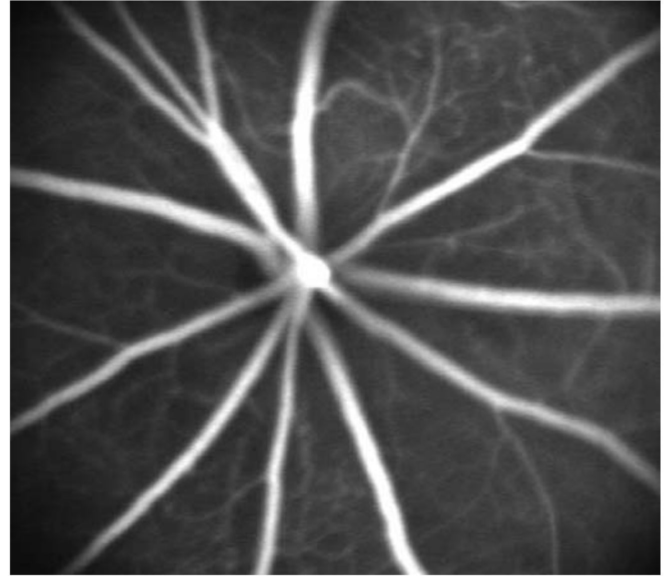

Fig. 6 Fluorescence fundus angiography image from the AGE-RSA-injected group

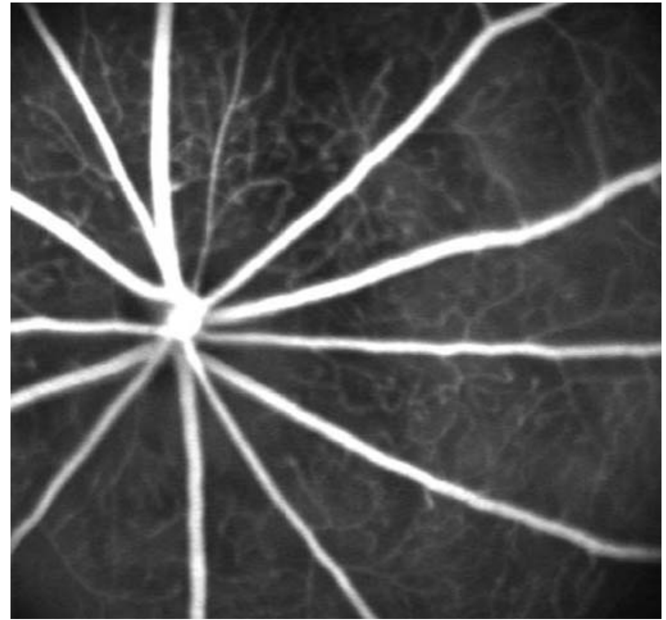

Fig. 7 Fluorescence fundus angiography image from the RSA-injected group

Immunohistochemical results

The retinae prepared from the AGE-RSA-injected group were all significantly positive for AGE immunohistochemical staining, in contrast to the negative staining in the other three groups (Table 1; Figs. 8, 9). Using the *H*

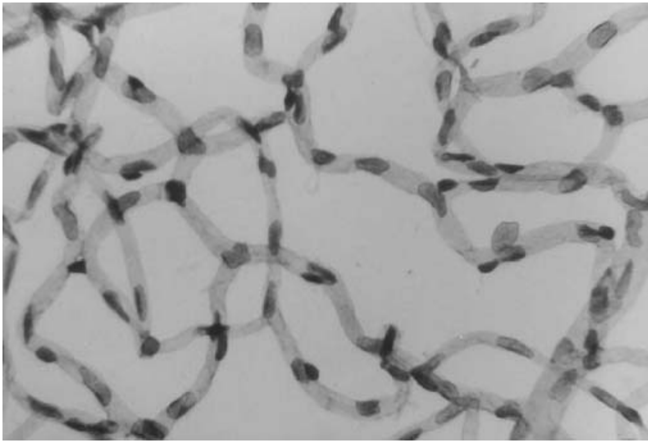

Fig. 8 AGE immunohistochemical staining of the retinal vascular tissue from the AGE-RSA-injected group (10×40 power field)

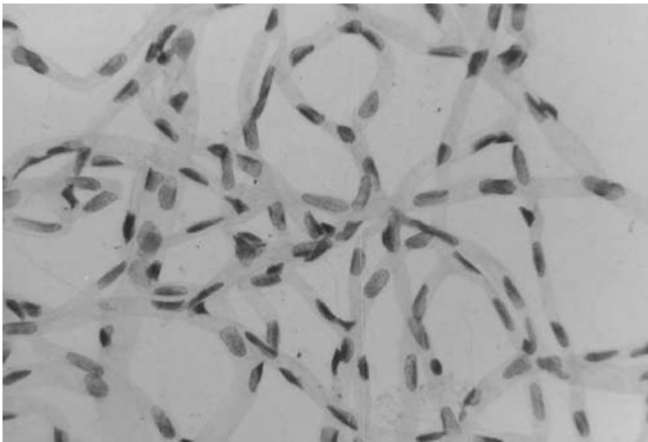

Fig. 9 AGE immunohistochemical staining of the retinal vascular tissue from the RSA-injected group (10×40 power field)

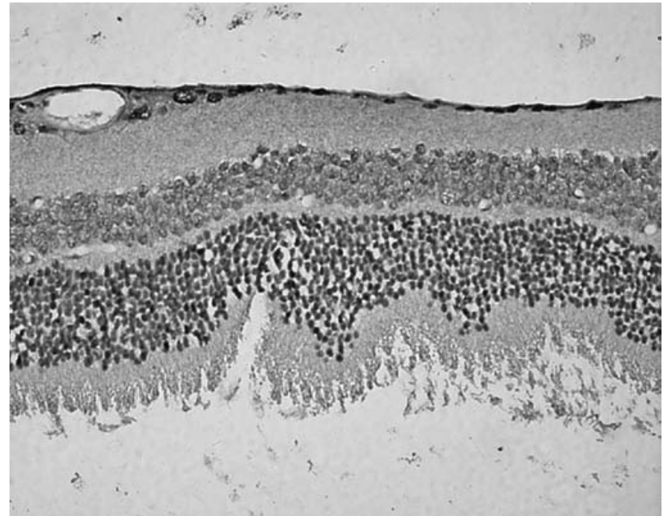

Fig. 10 Flt-1 immunohistochemical staining of the retina from AGE-RSA group (10×40 power field)

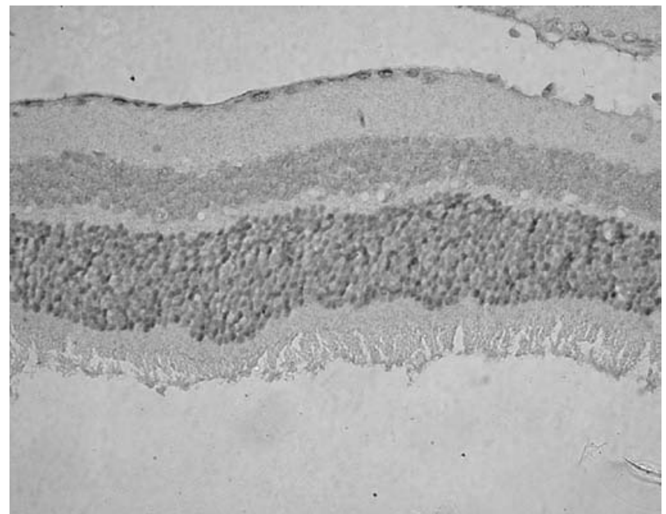

Fig. 11 Flt-1 immunohistochemical staining of the retina from the RSA group (10×40 power field)

test, the differences in AGE immunohistochemical staining, shown in Table 1, were statistically significant (H=15, P<0.01). The AGE-RSA-injected group also had more positive cells in the inner nuclear layer whose karyotheca dyed brown on Flt-1 immunostaining than the RSA-injected group in 10×40 power fields (AGE-RSA 25.18±3.44, RSA 18.74±4.11; P<0.05) (Figs. 10, 11).

Discussion

Advanced glycosylation end products are a group of irreversible end products of the Maillard serial reaction, from reductive glucose with proteins, lipids, or nuclear acids. AGEs are formed during this reaction, for example, by the binding of aldoses on free NH_2 groups of proteins, which, after a cascade of molecular rearrangements, result in molecules of brown color and specific fluorescence. Formation of AGEs is a normal physiological process that is accelerated under the hyperglycemic condition in diabetes. Under normal conditions, AGEs accumulate slowly with increasing age.

Diabetic patients have complicated metabolic abnormalities other than high blood glucose. It was hard for us to conclude that AGE deposition is one of the factors leading to the numerous pathologic changes including

diabetic retinopathy independently. In this study, the exogenous AGEs formed from RSA and D-glucose had physical and chemical characteristics typical of AGEs [10] and they immunoprecipitated with anti-AGE multiclonal antibodies [7]. It has been shown that AGEs can deposit on renal vascular tissues and cause dysfunction, including vascular dilation, lumen narrowing, and vascular leakage. However, there are so far few reports on their effects on retinal vascular changes. This study has demonstrated that exogenous AGEs, labeled by specific immunohistochemical staining, can deposit in vivo on retinal vascular tissues in non-diabetic rats, and thatthis also correspond to pericyte loss, mitochondrial swelling and elevated electronic density of the retinal basement membrane, which will probably increase retinal capillary leakage. These findings present a direct link between AGE deposition and early retinal vasculopathy.

Studies on retinal phenotype have shown that pericyte loss is among the early detectable changes in diabetic retinopathy [3]. Clinically, one of the early changes in diabetic retinopathy is edema, and fluoroscopy also shows local or general capillary leakage, while pericyte loss and dysfunction is one of the key factors. However we did not find capillary or choroidal dye leakage either in AGE-RSA group or in the RSA group after injection of the dye. Such apparently incompatible findings may be due to various factors which could contribute to prevent capillary leaking, such as the compensation period after capillary damage. Even though micromorphological changes were detected by transmission electron microscopy, gross alterations were not found in the course of short-term treatment. Retinal vascular leakage may be due to longer interaction between AGEs and the retina.

The finding of elevated numbers of Flt-1-positive cells in the AGE-RSA group implies that AGEs may have the ability of inducing VEGF receptor expression. Excess VEGF and its subsequent bonding to receptors may lead to vascular damage. AGEs may affect the retina by activating the VEGF pathway. It has been reported that AGEs and VEGF have somewhat similar distribution in retina [8], which may help explain this phenomenon.

AGEs alter the structure and function of molecules in biological systems and increase oxidative stress. These adverse effects of both exogenously and endogenously derived AGEs have been implicated in the pathogenesis of diabetic complications and changes associated with aging, including atherosclerosis, renal, and neurological disease. Specific AGE receptors and non-receptor mechanisms may contribute to these processes but also assist in the removal and degradation of AGEs. The receptor for AGEs (RAGE) is a member of the immunoglobulin superfamily. Experimental studies have indicated that the binding of AGEs to RAGE activates cells, particularly monocytes and endothelial cells. Activated endothelial cells produce cytokines and express adhesion molecules and tissue factor. AGEs are also reported to have a role in increased oxidative stress and in the functional alterations in vascular tone control observed in diabetes, in part related to a reduction in nitric oxide. Some authors have also reported that glycosylated proteins accumulated in vivo provide stable active sites for catalyzing the formation of free redicals directly [14].

Promising pharmacologic strategies to prevent AGE formation, reduce AGE toxicity, and/or inactivate AGEs are increasingly proving important and need further investigation.

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