Advanced Glycation End Products in Vitreous: Structural and Functional Implications for Diabetic Vitreopathy

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PURPOSE. Advanced glycation end products (AGEs) form irreversible cross-links with many macromolecules and have been shown to accumulate in tissues at an accelerated rate in diabetes. In the present study, AGE formation in vitreous was examined in patients of various ages and in patients with diabetes. Ex vivo investigations were performed on bovine vitreous incubated in glucose to determine AGE formation and cross-linking of vitreous collagen.

METHODS. By means of an AGE-specific enzyme-linked immunosorbent assay (ELISA), AGE formation was investigated in vitreous samples obtained after pars plana vitrectomy in patients with and without diabetes. In addition, vitreous AGEs were investigated in bovine vitreous collagen after incubation in high glucose, high glucose with aminoguanidine, or normal saline for as long as 8 weeks. AGEs and AGE cross-linking was subsequently determined by quantitative and qualitative assays.

RESULTS. There was a significant correlation between AGEs and increasing age in patients without diabetes ($r = 0.74$). Furthermore, a comparison between age-matched diabetic and nondiabetic vitreous showed a significantly higher level of AGEs in the patients with diabetes ($P < 0.005$). Collagen purified from bovine vitreous incubated in 0.5 M glucose showed an increase in AGE formation when observed in dot blot analysis, immunogold labeling, and AGE ELISA. Furthermore, there was increased cross-linking of collagen in the glucose-incubated vitreous, when observed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and protein separation. This cross-linking was effectively inhibited by coincubation with 10 mM aminoguanidine.

CONCLUSIONS. This study suggests that AGEs may form in vitreous with increasing age. This process seems to be accelerated in the presence of diabetes and as a consequence of exposure to high glucose. Advanced glycation and AGE cross-linking of the vitreous collagen network may help to explain the vitreous abnormalities characteristic of diabetes.

The nonenzymatic glycation of proteins by aldehyde or keto groups of reducing sugars in a process known as the Maillard reaction eventually causes the formation of advanced glycation end products (AGEs). AGEs have been implicated as causal factors in several aspects of the vascular complications of diabetes, including diabetic retinopathy, and acts in many cases through specific AGE receptor-mediated mechanisms. AGE moieties persist for the lifetime of the protein or lipid they are attached to, may display distinctive fluorescence, and have the ability to cross-link proteins to each other. Such irreversible cross-links persist on long-lived proteins and have been shown to accumulate with aging and at an accelerated rate in diabetes. Functionally, AGE cross-links in the collagen of extracellular matrix (ECM) are known to cause tissue rigidity, reduced solubility, and a decrease in the susceptibility of proteins to enzymatic digestion. Also, progressive AGE formation on lens crystallins accounts for a significant proportion of opacification of cataractous lenses in people with diabetes.

The vitreous is a hydrated gel matrix composed largely of a complex network of cross-linked collagen (types II, V, IX, and XI) fibrils and the hydrophilic glycosaminoglycan, hyaluronan. Many disorders of the vitreous manifest themselves as opacities in an optical structure that is normally transparent or in morphologic changes within the cortical vitreous. Often they are associated with aging changes affecting the anatomy and physiochemistry of the gel or with secondary changes caused by trauma, inflammation or other ocular diseases.
as a consequence of age (>50 years) the vitreous becomes detached from the retina and collapses anteriorly toward the pars plana of the ciliary body. Most vitreous degenerations are accompanied by liquefaction of the vitreous gel as a result of biochemical and physiological changes that cause dissociation of collagen and hyaluronan. Structural changes to the vitreous such as liquefaction and posterior vitreous detachment are associated with aging and also with the occurrence and onset of diabetic retinopathy. Moreover, patients with diabetes experience vitreous degeneration earlier than those without diabetes, which in turn may cause vitreoretinal traction and exacerbate the course of proliferative retinopathy. Little is known about the molecular basis for vitreous degeneration, but it has been demonstrated that abnormal cross-link formation in the collagen fibrils as a direct result of nonenzymatic glycation reactions causes dissociation of collagen from hyaluronan and results in destabilization of the vitreous. Although nonenzymatic glycation reactions ultimately cause AGE formation in long-lived proteins, little is known about AGEs and their effects in the vitreous, although Sebag et al. detected early and late glycation products in the vitreous of patients with advanced diabetic retinopathy. In the current investigation we examined vitreous samples obtained during vitrectomy from a large group of patients with diabetes and quantified the AGE moieties they contained. In a parallel ex vivo study, we investigated AGE formation on vitreous exposed to glucose and attempted to determine its influence in vitreous collagen cross-linking.

**Material and Methods**

**Vitreous Specimens**

The study was conducted according to the tenets of the Declaration of Helsinki. After obtaining the patients' consent, midvitreous samples were collected during pars plana vitrectomy operations in the eye clinic, Royal Victoria Hospital, Belfast, Northern Ireland. Pure, undiluted vitreous was removed (~500 μl volume) by using a three-tap port on the aspiration line that permitted removal before isotonic infusion and aspiration. Specimens were cataloged according to sex, age, and presence of diabetes (15 diabetic; 31 nondiabetic). Most of the patients were undergoing vitrectomy for diabetes (n = 15: 6 for intracortical vitreous hemorrhage and 9 for tractional detachment) or complex retinal detachment, including conditions such as macular holes (n = 5), epiretinal membrane (n = 5), trauma (n = 4), and subretinal neovascular membrane (n = 17). In all cases, those samples that contained high levels of blood contamination, estimated by obvious red or ochre coloration of the specimen, were excluded from the study. Specimens were snap frozen in liquid nitrogen in the operating theater and stored at −70°C until analysis.

**AGE Enzyme-Linked Immunosorbent Assay**

Undiluted vitreous sample was analyzed in a competitive AGE enzyme-linked immunosorbent assay (ELISA) using an AGE-bovine serum albumin standard curve. The assay was performed using a monoclonal AGE antibody, according to established protocols. Comparisons were made between patients' ages and the presence or absence of diabetes. Statistical comparison was made using a multivariate regression analysis and Student's t-test.

**Ex Vivo Experiments**

Bovine eyes were obtained from a local abattoir and the vitreous excised from the posterior segment under sterile conditions. After brief washing in sterile phosphate-buffered saline (PBS) to ensure that no contaminating tissue was present, a single undissected vitreous was placed into one of three incubation media: PBS containing 500 mM glucose, PBS containing 500 mM glucose with 10 mM aminoguanidine, or PBS without glucose. The specimens were incubated at 37°C for periods of 2 to 8 weeks with duplicate samples for each time period. After removal from the incubation medium, the vitrea were washed briefly in ice-cold PBS and dissected into quarters. Vitreous samples were placed in fresh ice-cold PBS containing protease inhibitors (5 μg/ml leupeptin, 2 mM phenylmethylsulfonyl fluoride, 10 μg/ml pepstatin, and 1 mM EDTA), homogenized in a tight homogenizer (Dounce; Kontes Glass, Vineland, NJ) and centrifuged (Optima TLX; Beckman, Palo Alto, CA) at 100,000g for 1 hour at 4°C. The samples were then treated as outlined by Akiba et al. Briefly, the insoluble pellet was resuspended in 20 volumes 1 M ethylenediamine dihydrochloride and agitated gently overnight at 4°C. The mixture was then centrifuged again at 100,000g for 30 minutes at 4°C. The soluble pellet was washed briefly in distilled water, freeze dried overnight in a tissue dryer (EDTA; Edwards, Crawley, UK), and weighed and the residue resuspended in 0.5 M acetic acid containing pepsin at 10 μg/100 μg per sample (Sigma, Poole, UK). This mixture was then left to digest slowly for 8 days at 4°C. After digestion the samples were centrifuged at 100,000g for 30 minutes at 4°C and the supernatant adjusted to pH 7.2. Solid NaCl was added to a final concentration of 4.4 M to precipitate the solubilized collagen after which the sample was centrifuged at 100,000g for 30 minutes at 4°C. The pellet was then resuspended in 0.5 M acetic acid and the solution dialyzed against distilled water in 0.5-ml dialysis cassettes (Pierce, Cheshire, UK). The dialyzed samples were then processed for AGE ELISA (as explained earlier) dot blot analysis, or cross-linking analysis.

**Dot Blot Analysis**

Samples of digested, dialyzed vitreous collagen were equalized according to protein content (micro-bicinchoninic acid [BCA] protein analysis, Pierce), and 10 μg was blotted onto nitrocellulose membrane (Bio-Rad, Hemel Hempstead, UK) using dot-blotting apparatus under gentle vacuum (Bio-Rad). After drying at room temperature, the blots were blocked in 1% bovine serum albumin and 0.1% Tween-20 in Tris-buffered saline and incubated with anti-AGE monoclonal antibody for 1 hour. After they were washed in Tris-buffered saline--Tweek, the blots were incubated in an anti-mouse peroxidase secondary antibody (Sigma). The blots were washed extensively and immunoactivity was detected with enhanced chemiluminiscence (Amersham, Amersham, UK).

**Vitreous Cross-Linking**

Equal amounts of vitreous collagen were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) under reducing conditions in 5% to 20% gradient gels (Bio-Rad). Gels were then subjected to silver staining according
The graph shows how AGEs occur at increasing levels in older patients without diabetes ($r = 0.74; P < 0.05$).

to the protocol of Heukeshoven and Dernick. The degree of cross-linking was determined relative to that in the normal (untreated) lanes by the density of the staining of high molecular weight protein at the top of the gel. The remainder of the digested vitreous samples were centrifuged in 100-kDa cutoff spin columns (Bio 101, Vista, CA) and the protein content contained in the less than 100-kDa fraction quantified by the micro-BCA method read in a 96-well spectrometer (SLT, Rainbow, Austria).

**Ultrastructural Demonstration of AGEs on Vitreous Collagen.**

The pellet from the initial vitreous separation, according to the protocol outlined earlier, was fixed in 0.5% glutaraldehyde–4% paraformaldehyde in PBS for 2 hours. The pellets were washed in ice-cold PBS, dehydrated, and embedded in resin (Unicryl; Bio Cell, Cardiff, UK). Sections were cut and floated onto gold-coated nickel grids and stained using an anti-AGE polyclonal antibody, according to the technique of Stitt et al. Briefly, the sections were blocked in normal goat serum, stained with anti-AGE RNase polyclonal primary antibody and subsequently with anti-rabbit 15-nm gold conjugated secondary antibody (Bio Cell). The AGE-RNase polyclonal primary antibody has been described previously. For each time period under investigation preabsorbed control experiments (AGE bovine serum albumin) and primary antibody omission control experiments were performed. In addition, anti-RNase control specimens were also used in which antibodies were raised against non-modified RNase.

**RESULTS**

**Patient Vitreous**

Forty-six vitreous samples were included in the analysis. Thirty-one of these were from patients without diabetes (age range, 1–83 years; mean, 47.76 years; median, 53 years) and 15 specimens were from patients with diabetes (age range, 39–81 years; mean, 57.5 years; median, 57.5 years).

**AGEs in the Nondiabetic Vitreous.** A multivariate regression analysis of nondiabetic vitreous data showed that there was a significant correlation of patient age and the AGE immunoreactivity of their vitreous ($r = 0.74$; Fig. 1). Patients were also divided into age tertiles, and AGE levels were compared (Fig. 2). Statistical analysis showed significant differences between the patient groups ($t$-test): 0 to 30 years versus 31 to 60 years ($P < 0.05$); 31 to 60 years versus 61 years or more ($P < 0.03$); 0 to 30 years versus 61 years or more ($P < 0.02$).

**AGEs in the Diabetic Vitreous.** For direct comparison with the sample of diabetic vitreous, the healthy specimens were obtained in patients in the 30 to 75 age range to match closely the mean age of the samples from patients with diabetes (see statistical comparison in Table 1 and Fig. 3). On analysis, there was a significant difference between AGE (shown in units per milliliter) when nondiabetic vitreous was compared with diabetic vitreous ($P < 0.0.005$).

**Ex Vivo Vitreous Investigations**

**Demonstration of AGEs in Vitreous Collagen.** In bovine vitreous incubated for increasing times in glucose, the AGEs were quantified by AGE-ELISA on vitreous samples from age-matched patients with and without diabetes who underwent vitrectomy.

**Ex Vivo Vitreous Investigations**

**Demonstration of AGEs in Vitreous Collagen.** In bovine vitreous incubated for increasing times in glucose, the

**Table 1. Comparison of the Advanced Glycation End Products Contained in Vitreous from Two Patient Groups**

<table>
<thead>
<tr>
<th>Patient Group</th>
<th>Age*</th>
<th>Age (U/ml)†</th>
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<tr>
<td>Nondiabetic ($n = 46$)</td>
<td>58.62 (58.01)</td>
<td>0.67 ± 0.062</td>
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<tr>
<td>Diabetic ($n = 15$)</td>
<td>58.08 (57.50)</td>
<td>2.56 ± 0.67</td>
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AGEs were quantified by AGE-ELISA on vitreous samples from age-matched patients with and without diabetes who underwent vitrectomy.

AGE, advanced glycation end products; ELISA, enzyme-linked immunosorbent assay.

* Values are means with medians in parentheses.
† Values are means ± SE.
FIGURE 3. Comparison of advanced glycation end product (AGE) immunoreactivity in vitreous specimens from patients with and without diabetes. Vitreous samples were matched according to patient age. There was a significant increase in AGEs in the diabetic vitreous. ***P < 0.005.

AGE ELISA of subsequently purified collagen indicated a significant increase in AGE levels when compared with levels in nonglucose control samples incubated for similar periods (Fig. 4A). Aminoguanidine did not reduce AGE levels in 2-week incubations. However, aminoguanidine seemed to reduce AGE formation significantly, in vitreous incubated for more than 5 weeks, although it failed to reduce AGE levels to nonglucose levels (Fig. 4A). Dot blot analysis of vitreous collagen extracts demonstrated a qualitative increase in AGE immunoreactivity between nonglucose and glucose-exposed vitreous (Fig. 4B).

There was a clear reduction in AGE immunoreactivity when vitreous was incubated in glucose in combination with 10 mM aminoguanidine (Fig. 4B).

Cross-Linking. SDS-PAGE of pepsin-digested vitreous collagen showed an increase in high molecular mass fragments (depicted by dense smearing of more than 120 kDa) after incubation in high-glucose conditions (Fig. 5A). This seemed to be reduced by coincubation with aminoguanidine (Fig. 5A). When the amount of collagen fragments of less than 100 kDa in pepsin-digested samples were quantified, there was a significant decrease in vitreous that had been incubated in high glucose, indicating a concomitant increase in cross-linking (P < 0.05; Fig. 5B). Coincubation with aminoguanidine decreased collagen cross-linking so that there was no significant difference between treated and control samples (Fig. 5B).

Ultrastructural Indication of AGES on Vitreous Collagen. Ultrastructural examination of the initial collagen-rich pellet showed the typical fibril appearance of the vitreous collagen network. A qualitative comparison of AGE immunoreactivity between control specimens and glucose-treated specimens at 8 weeks showed a considerably greater density of gold particles in the treated vitreous (Fig. 6A, 6B). Anti-RNase antibody incubations and negative control samples showed only sparse background immunoreactivity (Fig. 6C).

DISCUSSION

Intravitreal glucose has been shown to reflect blood glucose levels and can reach sufficient levels to permit intravitreal glycation reactions, especially in patients with diabetes. That nonenzymatic glycation may cause biochemical and functional abnormalities of diabetic vitreous has been reported through a demonstration of high levels of early glycation products in patients with diabetes when compared with patients without diabetes who undergo vitrectomy. In addition, fluorometric methods have shown 20 times higher levels of AGE moieties.

FIGURE 4. (A) Advanced glycation end product (AGE) levels in collagen extracted from vitreous incubated in phosphate-buffered saline (PBS); PBS and glucose; and PBS, glucose, and aminoguanidine for 2 or 5 weeks. There were significant increases in AGES in glucose-treated samples. Aminoguanidine significantly inhibited AGE formation at 5 weeks but not at 2 weeks. *P < 0.05; **P < 0.02. (B) Dot blots showing AGE immunoreactivity to a monoclonal anti-AGE antibody. There is a significant increase in staining intensity with exposure to glucose. This staining is effectively reduced by coincubation with aminoguanidine.
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in diabetic vitreous, accompanied by increased cross-linking of dihydroxylysylonorleucine on the vitreous collagen. Twenty times higher AGEs in diabetic vitreous compared with that in nondiabetic vitreous is very much higher than that observed in the current investigation. The reason for this apparent discrepancy may be that we meticulously excluded samples with extensive vitreous bleeding, thus ensuring that as few blood products as possible were incorporated into the assay. Moreover, differences may also be a consequence of the different AGE assay system used. The AGE ELISA is now the established method of quantifying AGE products in solutions and tissues and is currently used by several groups. Only some AGEs are fluorescently detectable, and the AGE ELISA has the advantage in that it can detect small levels of immunoreactive AGE moieties according to the primary antibody used. In the AGE ELISA in the present study, the same AGE antibody was used as that reported in previous investigations.

The positive correlation of AGEs in the vitreous collagen with increasing age in patients without diabetes is potentially important. AGEs have been shown previously to accumulate in many tissues with age, independent of diabetes, a process that can be inhibited by aminoguanidine. In contrast to cartilage collagen, it is now known that a component of the vitreous collagen shows active turnover, indicated by the presence of procollagen and immature cross-links in adult bovine vitreous. However, it remains likely that a large proportion of vitreous collagen is long lived and therefore subject to accumulative advanced glycation reactions.

The normally functioning vitreous provides visual transparency while maintaining the physiochemical properties of elasticit and viscosity. With age, the vitreous undergoes a liquefaction that is thought to be a result of dissociation of hyaluronan from the vitreous collagen network, possibly because of pooling of hyaluronan molecules into pockets of liquid vitreous as the collagen network becomes more aggregated and cross-linked. The current investigation indicates that AGEs form on the vitreous collagen network where they may cause AGE-derived cross-links possibly leading to age-related vitreous liquefaction. However, this aspect of the study has limitations, in that the specimens were obtained during vitrectomy after a posterior segment abnormality (e.g., ocular trauma), although in the absence of a systemic disease. It is unlikely that such abnormalities would alter AGE formation on vitreous collagen. Nonetheless, caution should be exercised in stating that AGEs increase in vitreous with aging. A more extensive study, examining an age range of eyes obtained after death would be required to confirm the current results. Sebag described the vitreous obtained after death from a range of patients of various ages using dark-field slit lamp microscopy and outlined the transition from gel homogeneity in children toward aggregation of collagen fibers in middle age, culminating in significant liquefaction in old age. The role of AGEs in
FIGURE 6. Immunogold labeling of advanced glycation end products (AGEs) on vitreous collagen. Vitrea were incubated in phosphate-buffered saline (PBS) only or in PBS containing glucose for 7 weeks. The vitreous collagen was subsequently extracted and pelleted. Sections were embedded in resin, and ultrathin sections were cut and stained with anti-AGE RNase or anti-RNase antibody and an appropriate 15-nm gold-conjugated secondary antibody. (A) Vitreous incubated in PBS showing AGE immunoreactivity on collagen. (B) Vitreous incubated in PBS and glucose for 7 weeks showing AGE immunoreactivity on collagen. (C) Vitreous incubated in PBS and glucose and stained with anti-RNase antibody. AGE immunoreactivity is significantly more abundant in glucose-incubated vitreous. Original magnification, X15,000.

this process should be fully elucidated, especially in that these structural changes are accelerated in diabetes. In the present study patients with diabetes showed a significantly higher level of advanced glycation than those without, which corresponds with previous findings using a different assay system. Increased AGES in serum and tissues is a characteristic of diabetes. A correspondingly high level of advanced glycation in the diabetic vitreous is important because reactive AGES may play an important role in the destabilization, premature liquefation, and complicated posterior vitreous detachment that occurs in many of these patients.

In the current investigation efforts were made to exclude vitreous samples obviously contaminated with blood by using exclusion criteria based on the gross red or ochre coloration of the specimen. However, it should be recognized that the remaining diabetic specimens may have had a history of vitreous hemorrhage to some degree, and we cannot therefore totally exclude the presence of blood-borne AGES in these vitreous samples. Although we acknowledge this, we emphasize that vitreous collagen is a long-lived molecule that accumulates AGES to a greater level than comparatively short-lived blood-borne molecules. It seems likely therefore, that AGE formation in the collagen network represents the bulk of AGE products in the vitreous specimens.

The ex vivo studies performed in the current investigation seemed to add further information to the AGE formation observed in the patient study. Purification of the collagen after the vitrea were incubated in glucose confirmed that AGES formed in this component of the vitreous gel. Because various AGES can form by a variety of chemical reactions according to environmental conditions, it remains difficult to make direct comparisons between AGE levels in the ex vivo and in vivo studies. It has in fact been demonstrated that exposure of proteins to high glucose concentrations in vitro can cause different AGE adducts to those formed under physiological conditions.

The increased proportions of high molecular weight collagen in glucose-incubated vitreous also shows the ability of AGES to cause cross-linking abnormalities in this structure. Aminoguanidine not only inhibited AGE formation but also seemed to prevent induction of cross-links. AGE-mediated cross-linking has been demonstrated in various nonvitreous collagen types in vivo and in vitro, a process that can be inhibited by aminoguanidine. The present results suggest that vitreous collagen is also subject to AGE-mediated cross-linking, because coinubcation of glucose and aminoguanidine can prevent pepsin-resistant cross-links and aggregates from forming. However, we cannot exclude the possibility that PAS-cross-link modifications such as N-ε-(carboxymethyl)lysine in the high-glucose incubations may inhibit proteolytic degradation and also affect the incidence of high molecular weight residues.

Abnormal cross-linking of vitreous collagen occurs after exposure to singlet oxygen or photoradiation. AGES have a well-recognized ability to generate free radicals during their formation, and it remains possible that a component of vitreous cross-linking is caused by oxidizing agents, perhaps in combination with advanced glycation.
This study describes the immunoreactive AGE-modified protein in the vitreous gel of patients with diabetes and of aging patients and supports the hypothesis that AGEs may have an important functional role in aging and diabetic vitreous complications. The pathogenesis of vitreous degeneration in patients with diabetes has been described as a process of precocious senescence. The current investigation suggests that the vitreous abnormalities observed in aging and diabetes may have a common pathogenic mechanism through the formation of AGE collagen cross-links. We now have the ability to prevent advanced glycation reactions through the use of aminoguanidine and related inhibitors. Such drugs may eventually find widespread use in patients with diabetes or in those at risk of age-related sequelae.

References