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Levels of Mature Cross-Links and Advanced Glycation End Product Cross-Links in Human Vitreous

Yukihiro Matsumoto*,
Masaaki Takahashi†, Makoto Chikuda* and Kiyomi Arai*

**Department of Ophthalmology, Koshigaya Hospital, Dokkyo University School of Medicine, Saitama, Japan; †Department of Orthopedic Surgery, Hamamatsu University School of Medicine, Shizuoka, Japan*

Purpose: To determine the levels of pyridinoline and deoxypyridinoline, two mature enzymatic cross-links, and pentosidine, an advanced glycation end product (AGE) cross-link, in the human vitreous, and to investigate the correlations among the cross-links and the effects of aging and diabetes mellitus (DM) on the levels of cross-links.

Methods: Forty-five vitreous samples were collected from 32 patients (32 eyes) undergoing vitrectomy for diabetic retinopathy (DM group) and from 13 patients (13 eyes) (control group) who were age- and sex-matched patients with idiopathic macular hole or epiretinal membrane with no systemic conditions. The levels of the cross-links were determined using high-performance liquid chromatography after acid hydrolysis and pretreatment with SP-Sephadex.

Results: The levels of pentosidine, pyridinoline, and deoxypyridinoline were 27.3 ± 23.1 (mean ± SD) pmol/mL (detectable in 45 of 45 specimens), 79.0 ± 40.2 ng/mL (43 of 45 specimens), and 54.0 ± 9.5 (32 of 45 specimens) ng/mL, respectively. When the vitreous samples from the DM and the control groups were compared, a significant difference ($P < .05$) was found in the pentosidine level but not in the levels of pyridinoline or deoxypyridinoline. No significant correlations were found between age and the cross-links. Significant correlations ($P < .01$) were found among the cross-links.

Conclusions: The results indicate that mature cross-link substances exist in the human vitreous. The results also suggest that glycation may occur in the vitreous after mature cross-links form and result in the formation of AGE cross-links. In human vitreous from patients with DM, increased levels of AGE cross-links may stabilize the formation of mature cross-links, but they did not increase the mature cross-links. **Jpn J Ophthalmol 2002;46:510–517** ©2002 Japanese Ophthalmological Society

Key Words: Advanced glycation end products, deoxypyridinoline, glycation, pentosidine, pyridinoline.

Introduction

Collagen, a primary constituent of the human vitreous, consists primarily of type II collagen that is similar to cartilage collagen and a small amount of type IX collagen. Each collagen molecule retains its elasticity and properties by cross-linkage.[1] Pyridinoline[2] and deoxypyridinoline,[3] the stable nonreducible mature cross-links formed under normal conditions, help maintain the structure of the collagen fibril network by contributing to the stabilization of the intermolecules of collagen.[4] Because they are excreted from the kidneys when collagen degrades, these two cross-links have been used widely as markers of bone metabolism, which reflects clinical bone resorption in orthopedics.[5–8]

Pyridinoline, extracted from the bovine Achilles tendon by Fujimoto et al,[2] and its minor analog, deoxypyridinoline, extracted from bovine bone by Ogawa et al,[3] are the pyridinium cross-links that belong to the 3- hydroxypyridinium family.[9] Pyridino-

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Correspondence and reprint requests to: Yukihiro MATSUMOTO, MD, Department of Ophthalmology, Koshigaya Hospital, Dokkyo University, 2-1-50, Minami-Koshigaya, Koshigaya, Saitama 343-8555, Japan

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line exists primarily in bone and cartilage and is present in small amounts in other tissues, such as tendon, fasciae, arteries, and is distributed in type I and II collagen; whereas deoxypyridinoline is in I and IV type collagen and bone and dentine exclusively, and to a lesser extent in most tissues.[6–8] To our knowledge, however, no studies have reported pyridinoline and deoxypyridinoline levels in the human vitreous.

Because the metabolic turnover of collagen, a long-lived protein, is slow, and because collagen contains substantial lysine residue, the accumulation of advanced glycation end products (AGEs) occurs frequently as the result of the nonenzymatic glycated reaction of the protein.[10–13] AGEs form disordered cross-links, resulting in changes in the structure and function of collagen.[10,14] Because glycation occurs more often in patients with diabetes mellitus (DM) compared with healthy subjects, a significant correlation between the accumulation of AGEs and the incidence and progress of diabetic complications has been reported.[15] Furthermore, AGEs are believed to be a factor in diabetic retinopathy, because growth factors and/or cytokines are produced through cell membrane receptors, which recognize AGEs in macrophages,[16] and these cytokines then cause intraocular proliferation and angiogenesis. In addition, AGEs form and accumulate to a greater extent in the vitreous of patients with diabetes, and the disordered AGE cross-links then form.[13,14]

In the present study, among the relevant AGEs, we focused on pentosidine, which forms the disordered nonenzymatic AGE cross-link by the ε-amino group of the lysine residue of the protein and reducing sugars, has cross-links, fluoresces, and is a stable material resistant to strong acid.[17] The amount of pentosidine in the tissue of patients with diabetes is significantly higher than in healthy subjects[11–13] because the level of serum pentosidine and the degree of diabetic retinopathy are proportional.[18] The amount of pentosidine in the vitreous of patients with diabetic retinopathy is significantly higher than in subjects with no diabetes.[13] Takahashi et al[5,8] developed a method to measure pentosidine, pyridinoline, and deoxypyridinoline in the hydrolysate using an SP-Sephadex column as pretreatment before high performance liquid chromatography (HPLC) with a fluorescence detector. This measurement detects the different dissolution times of the peak by a fluorescence detector, because their endogenous fluorescence spectra are different.

In the present study, we quantified the vitreous concentrations of pyridinoline and deoxypyridinoline, the mature lysyl oxidase-dependent cross-link components in collagen that are formed in an orderly fashion, as well as the vitreous concentrations of pentosidine, which changes the property of collagen by disordered cross-links of protein and accumulates in the vitreous of diabetic patients. We also analyzed the relation between the cross-links and aging and diabetes, which seems to affect cross-link generation.

Materials and Methods

Patients

Vitreous specimens were obtained from 32 diabetic patients (32 eyes; DM group), 38–76 years of age, mean age, 57.2 ± 13.2 years (mean ± SD) who underwent initial intraocular surgery at Dokkyo University Koshigaya Hospital for tractional retinal detachment plus vitreous hemorrhage (6 eyes/6 patients), tractional retinal detachment (3 eyes/3 patients), vitreous hemorrhage (18 eyes/18 patients), and diabetic macular edema (5 eyes/5 patients) complicating diabetic retinopathy. The mean hemoglobin (Hb)A1c value at surgery was 7.84 ± 1.64%, and the estimated duration of the diabetes was 10.3 ± 7.3 years. HbA1c was determined using an HPLC method that was operated on Hi-Auto A1c HA-8150 (Arkray, Kyoto). Vitreous specimens from healthy age- and sex-matched patients (range, 44–77 years; mean age, 64.7 ± 10.1 years) with no history of diabetes were used as the control. The absence of diabetes in the control group was determined by a fasting plasma glucose level < 126 mg/dL and an HbA1c value of 4.3–5.8%. Control specimens were obtained during the initial intraocular surgery from 13 patients (13 eyes) with idiopathic macular holes (7 eyes/7 patients) or epiretinal membranes (6 eyes/6 patients). Patients with levels of serum creatinine 1.3 mg/dL or higher were excluded.[12] Patients with bone disease, thyroid disease, and parathyroid disorders such as Paget's disease also were excluded.[19] Informed consent was obtained from each patient and each control subject before surgery.

Vitreous Specimen Collection

The vitreous samples were collected at the beginning of a three-port pars plana vitrectomy. A vitreous cutter was inserted into the midvitreous cavity before turning on the intraocular infusion to avoid contaminating the infusion solution; core vitrectomy then was performed under microscopic observation. An undiluted sample (approximately 1.0 mL) of vitreous gel was obtained by aspiration into a 2-mL

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sterile syringe attached to the vitreous cutter. The excised vitreous bodies were placed in an ice bath intraoperatively and immediately centrifuged (2,260 × *g* for 30 minutes at 4°C) to remove blood cells and proliferative tissue. The vitreous samples were stored at −40°C in a nitrogen-filled environment before use.

Quantification of Pentosidine in Vitreous Samples

Pentosidine was then quantified using the technique of Takahashi et al.[12] Excised vitreous humor was mixed with an equivalent amount of 12 mol/L HCl, degassed through a shield glass tube, and hydrolyzed for 20 hours at 110°C under anaerobic conditions produced by nitrogen gas replacement. Hydrolysates were filtered through a membrane filter with 0.45-μm pores (DIS-MIC-25 cs, Tokyo Roshi, Tokyo). Two hundred microliters of hydrolysates were diluted from the filtrate with 20 mL of deionized water and loaded onto a 0.8 × 1.0 cm SP-Sephadex C-25 column (Pharmacia LKB, Uppsala, Sweden). After washing with 20 mL of 0.1 mol/L HCl, the column was eluted with 5 mL of 1.0 mol/L HCl. The eluent was evaporated under reduced pressure using a TC-8 concentrator (Taitec, Tokyo). The residue was dissolved in 200 μL of 1.0% n-heptafluorobutyric acid (HFBA), and 160 μL of the diluents was analyzed using HPLC to determine the pentosidine content. The water was purified with a Milli-Q water purification system (Millipore, Bedford, MA, USA).

For the HPLC measurement, a Shimadzu HPLC system (Shimadzu, Kyoto) consisting of a SCL-6A system controller, LC-6A pump, SIL-10 auto injector, and RF-535 fluorescence HPLC monitor was used. The HPLC conditions were as follows: flow rate, 1 mL/min; mobile phase of acetonitrile and 40 mmol/L HFBA (27:73 by volume); ambient reaction temperature; an 8 mm × 10 cm column prepacked with Radial-Pack C18 (10-mm particle size, type 8C1810μ; Waters, Milford, MA, USA); and a fluorescence detector (excitation wave length, 335 nm; emission wavelength, 385 nm). The pentosidine standards of various concentrations were injected before and after a sample measurement in the same manner, and the standard curve was obtained.

Quantification of Pyridinoline and Deoxypyridinoline Levels in Vitreous Samples

The measurements of pyridinoline and deoxypyridinoline were the same as pentosidine until injection into the HPLC column in a hydrolysate, using prefractionation with SP-Sephadex C-25, and the fluorescence at 390 nm then was measured at 297 nm excitation.[5,8] A pyridinoline/deoxypyridinoline HPLC calibrator (Metra Biosystems, Mountain View, CA, USA) was used as the standard. The standards of various concentrations were injected before and after a sample measurement in the same manner, and the standard curve was obtained. Pyridinoline and deoxypyridinoline fluoresce and are resistant to hydrolysis by strong acid as well as pentosidine; however, they differ from pentosidine only in the optimum wavelength. The advantage of measuring the cross-links by HPLC is that because the cross-links are measured separately using a C18 column after they are extracted, an objective measurement of the cross-links is obtained.[20] Because samples are hydrolyzed by strong acid, total (free plus bound forms) pyridinoline and deoxypyridinoline are measured in this study.

Statistical Analysis

The levels of pentosidine, pyridinoline, and deoxypyridinoline were compared between the group with diabetes and the controls, and also in relation to aging. Statistical analysis was conducted using the nonparametric Mann-Whitney *U* test. The correlations were evaluated using Pearson's correlation coefficients. *P* values below .05 were considered significant. The results are expressed as the mean ± SD.

Results

Pentosidine, Pyridinoline, and Deoxypyridinoline Levels in the Vitreous of Patients with Diabetic Retinopathy and Control Eyes

Pentosidine was detected in 45 of 45 samples (from 32 samples from patients with diabetes and from 13 samples from control eyes), as compared with pyridinoline that was detectable in 43 of 45 samples and deoxypyridinoline that was detectable in 32 of 45 samples. The levels of pentosidine, pyridinoline, and deoxypyridinoline were 27.3 ± 23.1 pmol/mL (n = 45), 79.0 ± 40.2 ng/mL (n = 43), and 54.0 ± 9.5 ng/mL (n = 32), respectively. Vitreous samples from eyes with diabetes had a significantly higher level of pentosidine (31.4 ± 25.2 pmol/mL [n = 32]) than the control group (17.5 ± 13.0 [n = 13]; $P < .05$) (Figure 1). There was no significant difference in the vitreous levels of pyridinoline and deoxypyridinoline between the groups with diabetes (79.9 ± 44.1 ng/mL [n = 31], 55.7 ± 10.4 ng/mL [n = 20], respectively) and the controls (76.7 ± 21.3 [n = 12], 51.3 ± 7.6 [n = 12], respectively) (Figure 2).

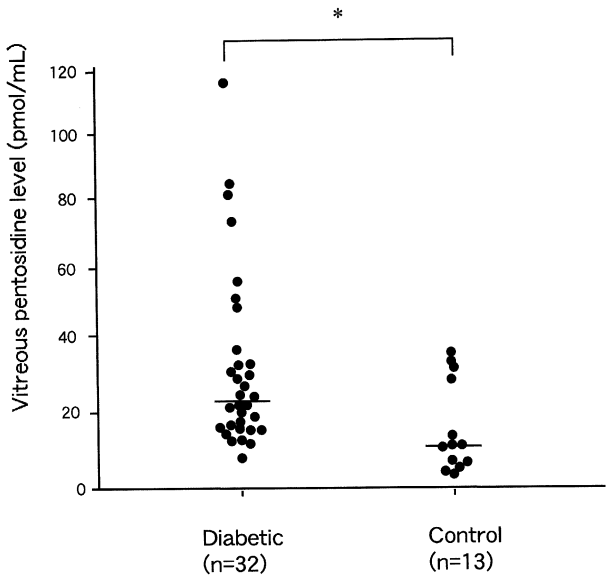

Figure 1. Pentosidine levels in vitreous samples from patients with diabetes and controls. The intravitreal levels of pentosidine are significant higher in the diabetic group than in the control group (Mann-Whitney *U*-test: $*P < .05$). n: number of samples.

Relation Between Pentosidine, Pyridinoline, and Deoxypyridinoline and Aging

There were no significant correlations between intravitreous pentosidine, pyridinoline, and deoxypyridinoline and age: $r = 0.12$ ($P = .52$), $r = 0.16$ ($P = .39$), and $r = -0.06$ ($P = .75$), respectively.

In the group with diabetes, the relations among the levels of pentosidine, pyridinoline, and deoxypyridinoline and age were $r = 0.29$ ($P = .22$), $r = 0.28$ ($P = .24$), and $r = 0.06$ ($P = .80$), respectively. Although the regression formulas of the pentosidine and pyridinoline levels gradually increased with age, no correlation was observed (Figures 3, 4, 5). In the control group, the correlations among the levels of pentosidine, pyridinoline, and deoxypyridinoline and age were $r = 0.07$ ($P = .84$), $r = -0.28$ ($P = .38$), and $r = -0.09$ ($P = .77$), respectively. There was no statistical correlation between the vitreous levels of pyridinoline and aging whereas they tended to decrease with age (Figure 4). There was no effect of age on the deoxypyridinoline levels in either the diabetes group or the controls (Figure 5).

Relation Among the Vitreous Levels of Pentosidine, Pyridinoline, and Deoxypyridinoline

The correlation coefficients were $r = 0.54$ for pentosidine and pyridinoline ($P < .01$), $r = 0.46$ for pentosidine and deoxypyridinoline ($P < .01$), and $r = 0.45$ for pyridinoline and deoxypyridinoline ($P < .01$). As shown in Figures 6, 7, and 8, significant correlations were observed between the vitreous levels of pentosidine and pyridinoline, pentosidine and deoxypyridinoline, and pyridinoline and deoxypyridinoline in the diabetes group ($r = 0.56$, $P < .01$; $r = 0.46$, $P < .05$; and $r = 0.54$, $P < .05$, respectively).

In the control eyes, no significant correlation was found among the levels of pentosidine and pyridinoline, pentosidine and deoxypyridinoline, and pyridi-

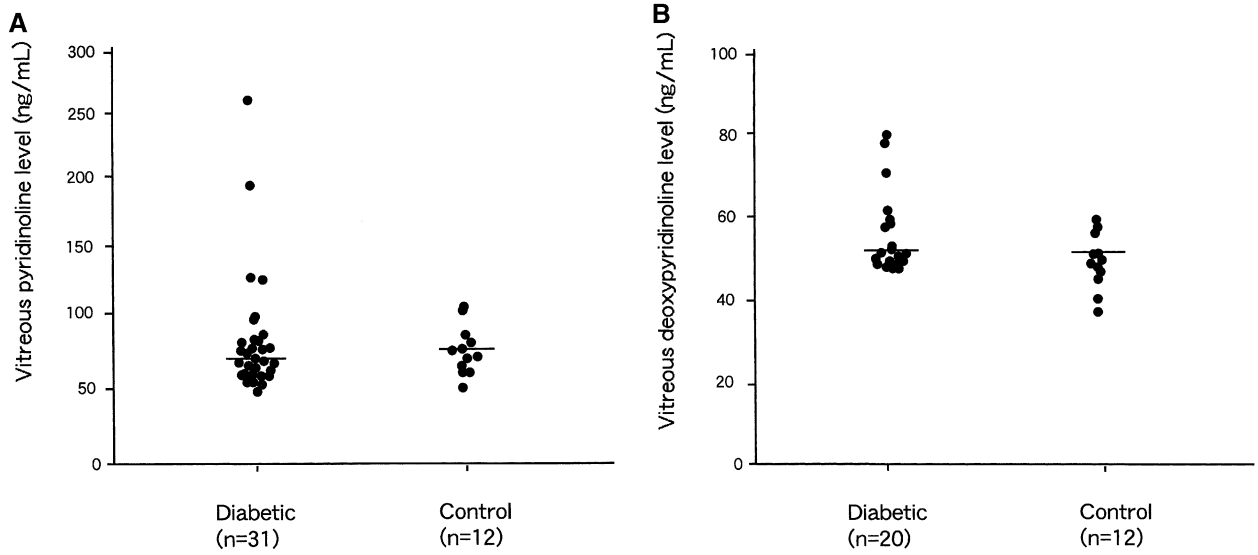

Figure 2. Pyridinoline and deoxypyridinoline levels in vitreous samples from patients with diabetes and controls. (**A**) Pyridinoline. (**B**) Deoxypyridinoline. No statistically significance differences are seen in the intravitreal level of pyridinoline and deoxypyridinoline between the diabetic and control groups (Mann-Whitney *U*-test). n: number of samples.

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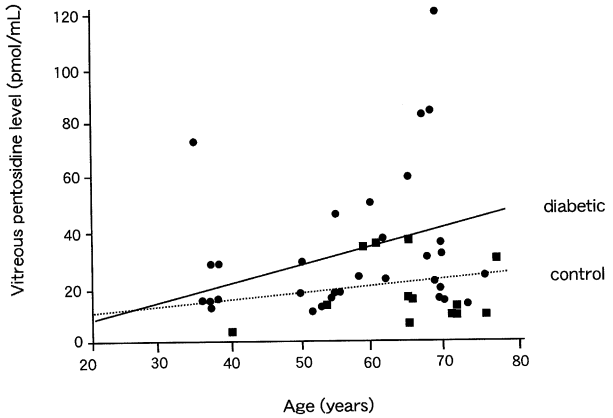

Figure 3. Correlations between intravitreal pentosidine and aging. Diabetes group (•): pentosidine (pmol/mL) = 0.412 × (age; years) + 7.84 (r = 0.29, P = .22, Pearson's correlation coefficients). Control group (■): pentosidine (pmol/mL) = 0.099 × (age; years) + 10.8 (r = 0.07, P = .84, Pearson's correlation coefficients). The solid line was obtained by a linear regression procedure applied to diabetes group cross-link level data; the dotted line represents control group data. No correlation is seen in the control group.

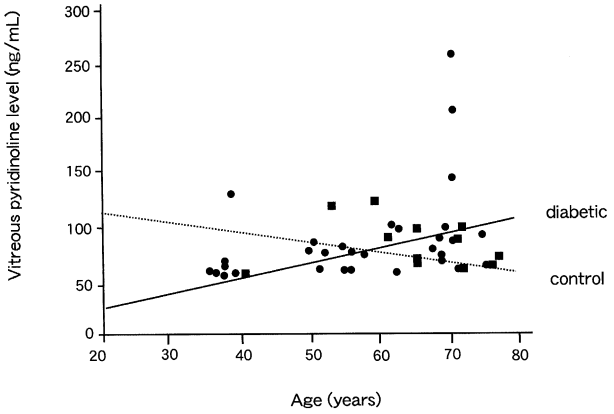

Figure 4. Correlations between intravitreal pyridinoline and aging. Diabetes group (•): pyridinoline (ng/mL) = 0.975 × (age; years) + 24.3 (r = 0.28, P = .24, Pearson's correlation coefficients). Control group (■): pyridinoline (ng/mL) = −0.582 × (age; years) + 114.3 (r = -0.28, P = .38, Pearson's correlation coefficients). The line indicates the linear regression equation [diabetes (solid line); control (dotted line)].

noline and deoxypyridinoline (r = 0.56, P = .06; r = 0.26, P = .41; r = 0.08, P = .82, respectively). However, there was a positive correlation, which was equivalent to the group with diabetes, between pentosidine and pyridinoline (Figure 6).

Discussion

Human vitreous pentosidine levels were significantly higher in the diabetic group (105.6 ± 56.1 pmol/vitreous in eyes with proliferative diabetic retinopathy vs. 40.4 ± 23.8 pmol/vitreous in eyes with idiopathic macular hole or ocular injuries without systemic diseases; n = 7 for each group).[13] In addition, the level of pentosidine in human cataractous lenses in patients with diabetes and those without were 169 ± 101 and 96.7 ± 73.6, respectively, suggesting that pentosidine is one of the causative factors of cataract.[13] The present study showed higher vitreous pentosidine levels in the diabetic group compared with the control group (Figure 1), confirmed advanced glycation reactions in the vitreous of patients with diabetes. This is thought to have occurred because proteins such as collagen undergo glycation by reduced sugars like glucose that are abundant in the vitreous of patients with diabetes and at the same time serum proteins flow into vitreous due to enhanced vascular permeability.[21] Further, we quantified the levels of two mature cross-links, pyridinoline and deoxypyridinoline, in the same samples and then clarified that these two substances exist in the human vitreous, so that significant correlations (r = 0.45, P < .01) were observed between the vitreous levels of the two mature cross-links. The levels of the mature cross-links were slightly higher in the diabetic group, but no significant difference was seen between levels in the diabetic and the control groups (Figure 2).

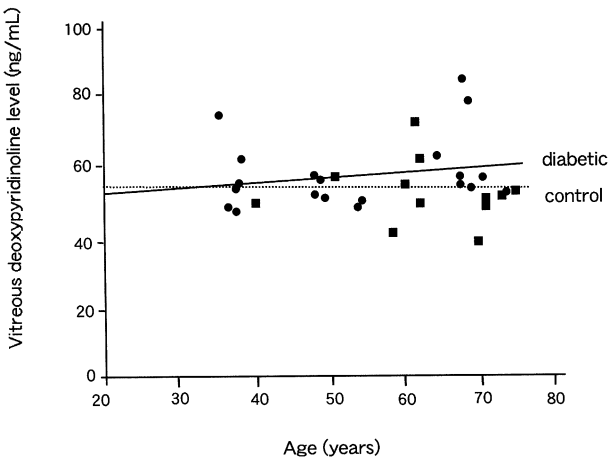

Figure 5. Correlations between intravitreal deoxypyridinoline and aging. Diabetes group (•): deoxypyridinoline (ng/mL) = 0.043 × (age; years) + 53.3 (r = 0.06, P = .80, Pearson's correlation coefficients). Control group (■): deoxypyridinoline (ng/mL) = −0.068 × (age; years) + 55.7 (r = -0.09, P = .77, Pearson's correlation coefficients). The line indicates the linear regression equation [diabetes (solid line); control (dotted line)].

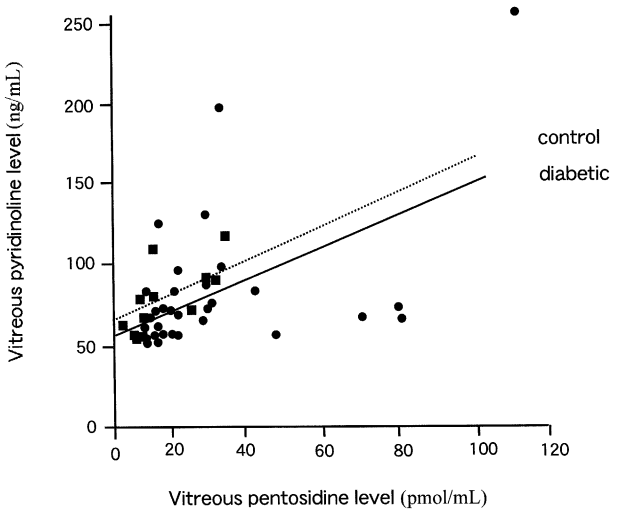

Figure 6. Correlations between intravitreal pentosidine and pyridinoline. Diabetes group (•): pyridinoline (ng/mL) = 0.822 × (pentosidine; pmol/mL) + 54.7 (r = 0.56, $P < .01$, Pearson's correlation coefficients). Control group (■): pyridinoline (ng/mL) = 0.869 × (pentosidine; pmol/mL) + 61.5 (r = 0.56, P = .06, Pearson's correlation coefficients). The line indicates the linear regression equation [diabetes (solid line); control (dotted line)].

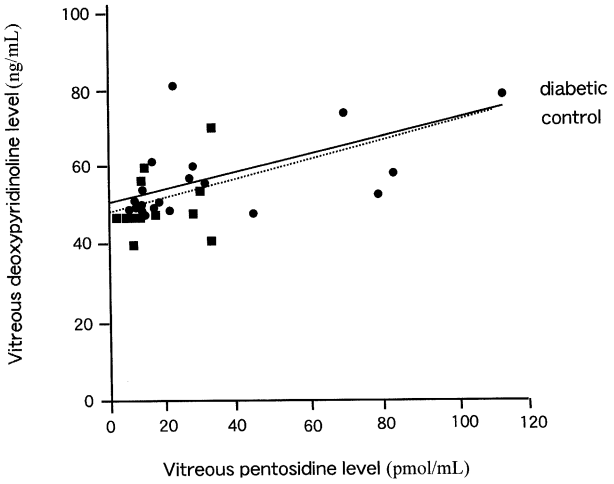

Figure 7. Correlations between intravitreal pentosidine and deoxypyridinoline. Diabetes group (•): deoxypyridinoline (ng/mL) = 0.157 × (pentosidine; pmol/mL) + 50.4 (r = 0.46, $P < .05$, Pearson's correlation coefficients). Control group (■): deoxypyridinoline (ng/mL) = 0.183 × (pentosidine; pmol/mL) + 48.1 (r = 0.26, P = .41, Pearson's correlation coefficients). The line indicates the linear regression equation [diabetes (solid line); control (dotted line)].

The levels of pyridinoline and deoxypyridinoline in human tissue after maturation are not related to aging,[6,22,23] and a similar observation was reported for the levels in murine tissues.[24] We also did not observe a correlation between these levels and aging (Figures 4 and 5), and thus it was surmised that aging has less effect on the formation of pyridinium cross-links in the vitreous. There also was no significant correlation between these levels and the presence of diabetes, but pyridinoline tended to accumulate in the vitreous in the diabetic group and to be lower with age in the control group (Figure 4) This decreasing tendency observed in the control group may be because the vitreous collagen level does not change substantially after 30 years of age[1] and because the lysyl oxidase activity generally decreases with age,[25] while it has been reported that pentosidine levels in human tissues[9,26] and in serum[12] increase with age. In the present study, the pentosidine level tended to gradually increase with age in both the diabetic and the control groups, but no significant correlation was observed between age and the level of each cross-link (Figure 3). This may have been partly the result of the fact that a fair percentage of the population of this study had diabetes. Further evaluation is needed in studies in which there are more controls 60 years of age or younger.

When newly formed collagen in the cell is incorporated into extracellular matrix and between the collagen fibers, cross-links are formed. At first, the cross-links are unstable, reducing, Schiff base-like cross-links, and then they are transformed into nonreducing mature pyridinium cross-links as they mature. Pyridinium cross-links originate from mature matrix and are formed only between extracellular mature collagen fibers, not between premature collagen or new single topocollagen that cannot form fiber.[7] Also, they are normal cross-links that are formed in an orderly fashion. Pyridinoline and deoxypyridinoline have three amino groups that facilitate their contribution to the intra- and intermolecular stabilization of adjacent collagen molecules. In other words, the formation of pyridinium cross-links requires substrates for lysyl oxidase such as lysine and hydroxylysine residues in intra- or intercollagen molecules, and begins with the substitution of an ϵ-amino group in the lysine residue of mature collagen with an aldehyde group by lysyl oxidase. The ϵ-amino group in the lysine residue, however, is also the target site for the glycation reaction of proteins such as vitreous collagen. Therefore, if glycation occurs in the same lysine residues before the reaction by lysyl oxidase during the process of collagen maturation, they would form Amadori substances, which leads to the inhibition of normal formation of cross-links.[22] On the other

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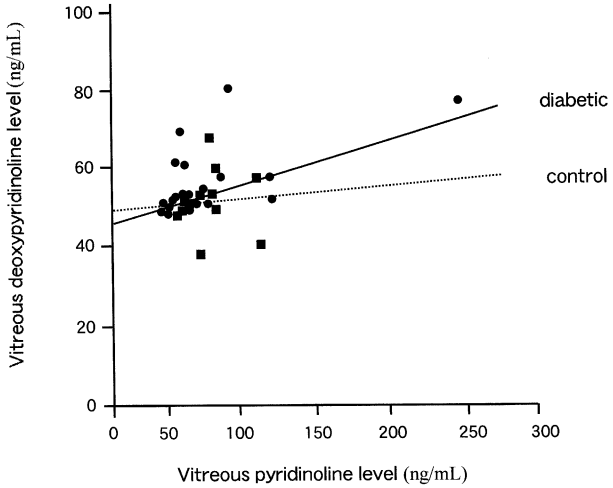

Figure 8. Correlations between intravitreal pyridinoline and deoxypyridinoline. Diabetes group (•): deoxypyridinoline (ng/mL) = 0.118 × (pyridinoline; ng/mL) + 46.2 (r = 0.54, $P < .05$, Pearson's correlation coefficients). Control group (■): deoxypyridinoline (ng/mL) = 0.027 × (pyridinoline; ng/mL) + 49.2 (r = 0.08, P = .82, Pearson's correlation coefficients). The line indicates the linear regression equation [diabetes (solid line); control (dotted line)].

hand, the level of pyridinoline increases together with the level of AGE cross-links in the skin of patients with diabetes.[27]

From these findings, we developed two hypotheses regarding the relation among the cross-link substances in the vitreous of patients with diabetes: (1) AGE cross-link formation increases and pyridinium cross-link formation decreases because the ε-amino groups of the lysine residues that are required for normal collagen formation undergo glycation by reduced sugars before pyridinium cross-link formation, or (2) AGE cross-link (such as pentosidine) formation increases and pyridinium cross-link formation remains at the same level or increases because the late stage of nonenzymatic glycated reactions are enhanced, with lysine residues that are not involved in pyridinium cross-link formation undergoing glycation after pyridinium cross-link formation.

In this study, because a significant ($P < .01$) positive correlation was observed between the levels of the pyridinium cross-links and the AGE cross-link in the vitreous in the diabetes as well as the control groups, the AGE cross-link and pyridinium cross-links may be closely related to each other, because the fluctuation of their levels is associated with each other.[27] Also, increased AGE cross-link formation in the vitreous may be more likely to occur after the formation of pyridinium cross-links, as described in hypothesis 2, rather than as the result of the glycation in the process of pyridinium cross-link formation, as described in hypothesis 1. In addition, glycation was considered to occur in the lysine residue where pyridinium cross-links are not yet formed.

In the diabetic group, significant correlations were seen between pentosidine and pyridinoline (r = 0.56, $P < .01$), between pentosidine and deoxypyridinoline (r = 0.46, $P < .05$), and between pyridinoline and deoxypyridinoline (r = 0.54, $P < .05$) (Figures 6, 7, and 8). In the control group, although no significant difference in the vitreous levels of these substances was observed, a positive correlation with a similar correlation coefficient (r = 0.55) was seen between pentosidine and pyridinoline. The levels of cross-link substances converged near the origin (the intersection of the x and y axes) in the control group, while they ranged widely over higher levels in the diabetic group. However, the regression curves for both groups were similar (Figure 6). Because an analogous tendency was observed for the deoxypyridinoline and pentosidine levels (Figure 7), the pentosidine cross-link and the pyridinium cross-link may be formed in a similar process that is irrelevant to the presence of diabetes. We considered that the vitreous pyridinium cross-link levels and the distributions of pyridinoline and pentosidine levels were slightly higher in the diabetic group compared with the control group, because the lysyl oxidase activity may be enhanced in the diabetic group. Pyridinium cross-links became resistant to metabolism and the half-life was extended, because AGE cross-links are formed after, rather than before, the pyridinium cross-links in the diabetic group compared with the control group, as described previously.

In this study, increased levels of the AGE cross-link were more frequently observed in the vitreous of patients with diabetes, and we hypothesized that pyridinoline accumulates in the vitreous as the result of delayed metabolism and its long half-life, because the emergence of the collagen fiber, which abounds in irregular AGE cross-links, enhances protein insolubility and their resistance to proteases.[16,28] The accumulation of collagen in the vitreous, which increases the chance of glycation, also may trigger a vicious cycle; promotion of late-stage reactions, and the further increase of AGE cross-links.

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Several references concerning the hypothesis presented in the *Nippon Ganka Gakkai Zasshi* version of the paper (*J Jpn Ophthalmol Soc*) 2002;106:9–15 have been deleted following the recommendations of the *JJO* reviewers of this paper.

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