Relationship between Glycoxidation and Cytokines in the Vitreous of Eyes with Diabetic Retinopathy

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Purpose: To investigate the correlations among pentosidine, an advanced glycation end product, and related cytokines (vascular endothelial growth factor [VEGF], transforming growth factor [TGF]-β_2, and monocyte chemotactic protein [MCP]-1), and active oxygen in the vitreous of patients with diabetic retinopathy (DR).

Methods: Vitreous samples from 43 eyes that underwent vitrectomy to treat DR were divided into four subgroups and analyzed. Vitreous samples from 21 eyes of patients with no systemic conditions served as age- and sex-matched controls. The vitreous levels of pentosidine, cytokines, and hydrogen peroxide (H_2O_2) were determined by high-performance liquid chromatography, enzyme-linked immunosorbent assay, and the titanium-hydrogen peroxide colorimetric method, respectively.

Results: The levels of pentosidine, VEGF, total TGF-β_2, and MCP-1 in the vitreous samples of eyes with DR were significantly higher than in the controls ($P < .01$). Significant correlations ($P < .01$) were found between pentosidine and VEGF, pentosidine and H_2O_2, H_2O_2 and VEGF, and total TGF-β_2 and MCP-1 ($r = 0.62$, 0.58, 0.65, and 0.59, respectively).

Conclusions: These results suggest that elevated levels of pentosidine may cause increased levels of VEGF in the vitreous of patients with DR. Increased levels of total TGF-β_2 and MCP-1 together also might play an important role in the development of DR. **Jpn J Ophthalmol 2002;46:406–412** © 2002 Japanese Ophthalmological Society

Key Words: Diabetic retinopathy, glycoxidation, pentosidine, transforming growth factor-β_2, vascular endothelial growth factor.

Introduction

Glycation is a chemical reaction in which proteins are nonenzymatically glycated in vivo,[1] and glycoxidation is the later stage of glycation in which advanced glycation end products (AGEs) form as the result of participation of oxidation in in vivo reactions.[2] Although pentosidine,[3–6] carboxymethyllysine (CML),[7,8] pyrraline,[9] and crosslines[10] are the major AGEs thus far identified, there is concern about glycoxidation in the generation of only pentosidine and CML.[2,11,12] Among the relevant AGEs, pentosidine fluoresces, has crosslinks, and is fairly resistant to strong acid, and several studies have reported that pentosidine accumulates in the ocular tissues of patients with diabetes mellitus (DM).[1,2,5,6]

Superoxides that form during the AGE-generating process alter hydrogen peroxide (H_2O_2) by dismutation,[13] and superoxides may cause greater accumulation of pentosidine. According to previous reports, AGEs bind to functional receptors in macrophage and vascular cells, and macrophages produce growth factors (cytokines).[8] Although these cytokines and growth factors are involved in intraocular angiogenesis and proliferation, to the best of our

knowledge, no reports have been published about the relation between glycoxidation and cytokines in the same vitreous in eyes with diabetic retinopathy (DR). In the present study, we quantified the vitreous levels of pentosidine, H_2O_2, and monocyte chemotactic protein (MCP)-1, which have potent chemoattractant properties against monocytes and macrophages, transforming growth factor (TGF)-β_2, and vascular endothelial growth factor (VEGF).[14,15] We also analyzed the relation between glycoxidation and cytokines in relation to the preoperative retinal complications of DR.

Materials and Methods

Patients

Vitreous specimens were obtained from 43 eyes of 40 patients (DR group, mean age = 57.7 $\pm$ 11.3 [mean $\pm$ SD] years) that underwent the initial intraocular surgery at Dokkyo University Koshigaya Hospital for tractional retinal detachment (TRD) plus vitreous hemorrhage (VH) (12 eyes of 11 patients), TRD (6 eyes), VH (18 eyes of 17 patients), and diabetic macular edema (DME) (7 eyes of 6 patients) complicating DR.[16] The patients included in the TRD and DME groups were limited to those in whom we judged that VH was not present for over 6 months preoperatively in our follow-up or in the inquiries. The clinical findings of patients who had TRD were divided as follows: in the macular TRD plus VH group, macular TRD (4 eyes), extramacular TRD (7 eyes), and macular TRD with a retinal break (1 eye); and in the TRD group, macular TRD (6 eyes).

Age- and sex-matched vitreous specimens from patients (mean age $\pm$ SD = 61.2 $\pm$ 11.8 years) without other ocular and systemic conditions served as the control group. Specimens were obtained during the initial intraocular surgery from 21 eyes of 21 patients, who were diagnosed with idiopathic macular hole or epiretinal membrane. The mean hemoglobin (Hb) A1c value at the time of the surgery was 7.53 $\pm$ 1.79% (mean $\pm$ SD), and the estimated duration of the DM was 10.50 $\pm$ 7.84 years (mean $\pm$ SD). Patients with abnormal levels of serum creatinine (1.3 mg/dL or more) were excluded from this study.[4]

Collection of Vitreous Specimens

The vitreous samples were collected at the beginning of a three-port pars plana vitrectomy in the same manner. A vitreous cutter was inserted into the midvitreous cavity before turning on the intraocular infusion to avoid contaminating the infusion solution, and then core vitrectomy was performed under microscopic observation in all cases. An undiluted sample (approximately 1.0 mL) of vitreous gel was collected by aspirating the gel into a 2-mL sterile syringe attached to the vitreous cutter. The excised vitreous bodies were placed in an ice bath intraoperatively and immediately centrifuged (2,260 $\times$ 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. Informed consent was obtained from each patient before surgery.

Quantification of Pentosidine in Vitreous Samples

Pentosidine was then quantified using the technique described by Takahashi et al.[4] A vitreous sample 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 $\times$ 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 high-performance liquid chromatography (HPLC) to determine the pentosidine content. The water used was purified with a Milli-Q water purification system (Millipore, Bedford, MA, USA).

For the HPLC measurement, a Shimadzu HPLC system (Shimadzu, Kyoto) was used that consisted of a SCL-6A system controller, LC-6A pump, SIL-10 auto injector, and RF-535 fluorescence HPLC monitor. 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 $\times$ 10 cm column prepacked with Radial-Pack C18 (10-mm particle size, type 8C1810μ; Waters, Milford, MA, USA); and a fluorescent detector (excitation wave length, 335 nm; emission wavelength, 385 nm). The pentosidine standards of various concentrations, ranging from 1.33 to 53 pmol, were injected before and after a sample measurement in the same manner, and the standard curve was obtained.

Quantification of TGF-β₂, VEGF, and MCP-1 Levels in Vitreous Samples

The levels of TGF-β_2, VEGF, and MCP-1 were determined by an enzyme-linked immunosorbent assay (ELISA) technique using Quantikine (R&D Systems, Minneapolis, MN, USA). TGF-β_2 is considered to be the predominant TGF-βs isoform in the vitreous.[14] Thus, the levels of total (mature form plus latent form) and mature TGF-β_2 were assessed in this study. Activation of samples for total TGF-β_2 was achieved by adding 25 μL of 1 mol/L HCl to 125 μL of a sample, incubating it at an ambient temperature for 10 minutes, and reacting it with 25 μL of a mixed solution of 1.2 mol/L NaOH and 0.5 mol/L HEPES, and 800 μL of buffered protein base solution. In addition, the VEGF kit was specific for VEGF$_{165}$, which is mostly produced in vivo. The procedures were performed according to the instructions of the manufacturer.

The standard curve was obtained with recombinant human TGF-β_2 (ranging from 15.6 to 2000 pg/mL), with recombinant human VEGF (ranging from 31.2 to 2000 pg/mL), and with recombinant human MCP-1 (ranging from 31.2 to 2000 pg/mL). The samples that exceeded 2000 pg/mL were measured again after dilution. The minimum detectable limit of TGF-β_2 was 7.0 pg/mL, and those of VEGF and MCP-1 were both 5.0 pg/mL. The optical density of each well was measured at 450 nm, with a correction wavelength of 570 nm (model MPR-A4, Toyo Soda, Tokyo). The mean value of the two measurements including the standard was calculated, and simultaneously the calibration curve was made and the calibration of the concentrations of VEGF, total TGF-β_2, mature TGF-β_2, and MCP-1 in each sample were determined.

Quantification of H₂O₂ in Vitreous Samples

H_2O_2 was quantified using a titanium-hydrogen peroxide colorimetric method.[17] Samples (80 μL) were mixed with an equivalent amount of 10 mmol/L phosphate buffer (pH 7.00). After mixing, the titanium colorimetric solution was added to each microplate well. The plate was incubated at 25°C for 90 minutes, and the optical density of each well was measured at 410 nm, with a correction wavelength of 570 nm. The standard curve was obtained for H_2O_2, ranging from 3.6 to 910 nmol/mL. The mean value of the two measurements including the standard was calculated, and simultaneously the calibration curve was made and the calibration of the H_2O_2 concentrations in each sample was determined.

Statistical Analysis

The levels of pentosidine, VEGF, total TGF-β_2, mature TGF-β_2, MCP-1, and H_2O_2 were compared among the DR groups and the control group. Statistical analysis was based upon the nonparametric Mann-Whitney U-test. The correlations were evaluated using Pearson correlation coefficients. P values below .05 were considered significant. The results are shown as the mean ± SD.

Results

Pentosidine Levels in the Vitreous of DR and Control Eyes

Vitreous from eyes with DR contained a significantly higher level of pentosidine (29.6 ± 18.9 pmol/mL) than the control group (10.2 ± 7.5 pmol/mL; P < .01).

Vitreous levels of pentosidine in relation to retinal complications of DR were 20.5 ± 11.2 pmol/mL in the TRD plus VH group, 29.6 ± 14.5 pmol/mL in the TRD group, 34.7 ± 24.6 pmol/mL in the VH group, and 27.9 ± 12.9 pmol/mL in the DME group. These results were significantly higher than in the control group (P < .01, Figure 1). The levels of intravitreous pentosidine did not differ among the four diabetic subgroups; however, the level of pentosidine in the VH group was the highest compared with controls (P < .0001).
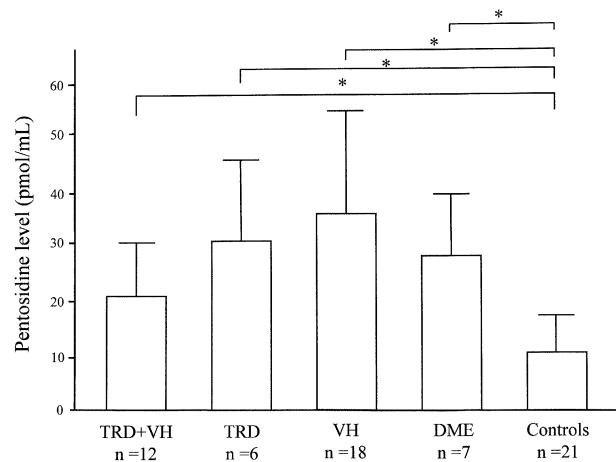

Figure 1. Pentosidine levels in vitreous from patients with diabetic retinopathy in the subgroups and controls. The intravitreal levels of pentosidine in the subgroups with tractional retinal detachment (TRD) plus vitreous hemorrhage (VH), TRD, VH, and diabetic macular edema (DME) are significantly higher than in the control group (Mann-Whitney U-test: *P < .01). n: number of samples.

Relation between Vitreous Levels of Pentosidine and Serum HbA1c in the DR Group

There was no statistical correlation between the vitreous levels of pentosidine and serum HbA1c ($r = 0.01$, $P = .93$). In the group with a preoperative VH (TRD plus VH group and the VH group; n = 30), the vitreous level of pentosidine was 30.2 ± 20.7 pmol/mL, and the serum level of HbA1c was $7.77 \pm 1.97\%$ ($r = -0.09$, $P = .66$). In the group with no VH preoperatively (TRD and DME groups; n = 13), the vitreous level of pentosidine was 28.7 ± 13.1 pmol/mL, and the serum level of HbA1c was $7.17 \pm 1.46\%$ ($r = 0.43$, $P = .19$). There was no significant difference between the vitreous pentosidine level and the serum HbA1c level in the presence or absence of VH.

VEGF, TGF-β_2, and MCP-1 Levels in Vitreous of DR and Control Eyes

VEGF was detected in 42 of 43 vitreous specimens from eyes with DR and in 14 of 21 control specimens. The VEGF level was significantly higher in eyes with DR than in control eyes (836 ± 687 pg/mL vs 215 ± 107 pg/mL; $P < .01$). The levels of total TGF-β_2 were significantly higher in eyes with DR than in control eyes (3488 ± 2526 pg/mL vs 1473 ± 908 pg/mL; $P < .01$). There was no difference in the level of mature TGF-β_2 between eyes with DR and control eyes (369 ± 305 pg/mL and 340 ± 179 pg/mL; $P = .60$). Moreover, the level of MCP-1 was significantly higher in eyes with DR than in control eyes (2979 ± 2085 pg/mL vs 1214 ± 994 pg/mL; $P < .01$).

Based on the retinal complications of DR, the VEGF levels were 924 ± 665 pg/mL in the TRD plus VH group, 771 ± 714 pg/mL in the TRD group, 837 ± 765 pg/mL in the VH group, and 788 ± 616 pg/mL in the DME group ($P < .01$, $P < .01$, $P < .05$, and $P < .01$, respectively). The levels of MCP-1 (Figure 2) were 4093 ± 2011 pg/mL in the TRD plus VH group, 3823 ± 3054 pg/mL in the TRD group, and 2378 ± 1806 pg/mL in the VH group, which were significantly higher compared with the controls ($P < .01$, $P < .05$, and $P < .05$, respectively). The total TGF-β_2 level was 4247 ± 2885 pg/mL in the TRD plus VH group, 3459 ± 1882 pg/mL in the TRD group, and 3121 ± 1245 pg/mL in the DME group, which was significantly different compared with the controls ($P < .01$, $P = .01$, $P = .01$, respectively). No statistically significant difference was found in mature TGF-β_2 between the four diabetic subgroups and the controls.

The MCP-1 levels in the TRD plus VH group were significantly different than in the VH and DME groups ($P < .05$). When the vitreous levels of VEGF,
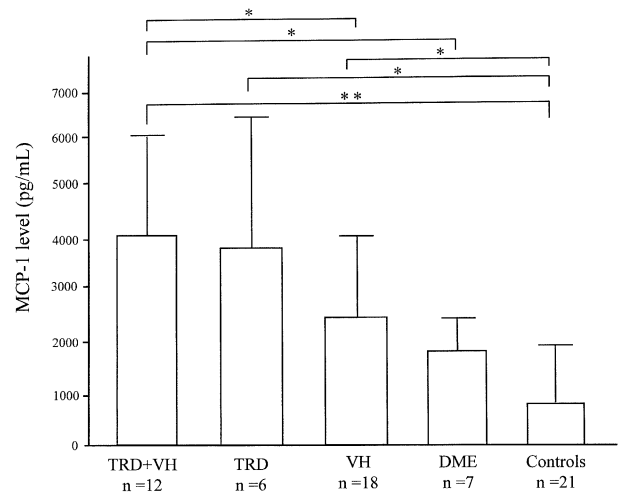

Figure 2. Monocyte chemotactic protein (MCP)-1 levels in vitreous from patients with diabetic retinopathy in the subgroups with tractional retinal detachment (TRD) plus vitreous hemorrhage (VH), TRD, VH, and diabetic macular edema (DME) and controls. The statistical significance was determined with the Mann-Whitney U-test. The MCP-1 level in the four diabetic groups: TRD plus VH: $**P < .01$ (vs control group), $*P < .05$ (vs VH, DME); TRD: $*P < .05$ (vs control group); VH: $*P < .05$ (vs control group) (Mann-Whitney U-test). n: number of samples.

total TGF-β_2, and mature TGF-β_2 were compared, there were no significant differences among the four subgroups.

H_2O_2 Levels in Vitreous in DR and Control Eyes

There tended to be higher levels of H_2O_2 in eyes with DR (12.8 ± 8.4 nmol/mL) compared with control eyes (detected in 19 of 21 eyes, 8.2 ± 4.6 nmol/mL).

Based on the retinal complications of DR, the vitreous levels of H_2O_2 in the TRD plus VH group, the TRD group, the VH group, and the DME group were 13.4 ± 8.0 nmol/mL, 11.7 ± 2.5 nmol/mL, 15.8 ± 9.8 nmol/mL, and 5.0 ± 2.5 nmol/mL, respectively. Statistically significant differences were found among the four groups, with the TRD plus VH group, the TRD group, and the VH group significantly higher than the DME group ($P < .05$, $P < .01$, and $P < .01$, respectively). The level in the VH group was significantly higher than in the control eyes ($P < .01$).

Relation Among the Vitreous Levels of Pentosidine, Cytokines, and H_2O_2

As shown in Figures 3, 4, and 5, there were significant correlations between the vitreous levels of
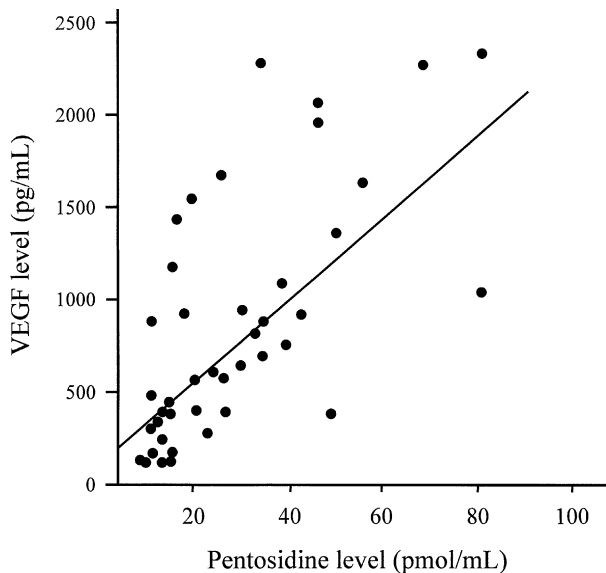
Figure 3. Correlations between intravitreal pentosidine and vascular endothelial growth factor (VEGF) in the groups with diabetic retinopathy. VEGF (pg/mL) = 22.42 × (pentosidine; pmol/mL) + 187.13 (r = 0.62, P < .01, Pearson correlation coefficients). The line indicates the linear regression equation. No correlation is seen in the control group.
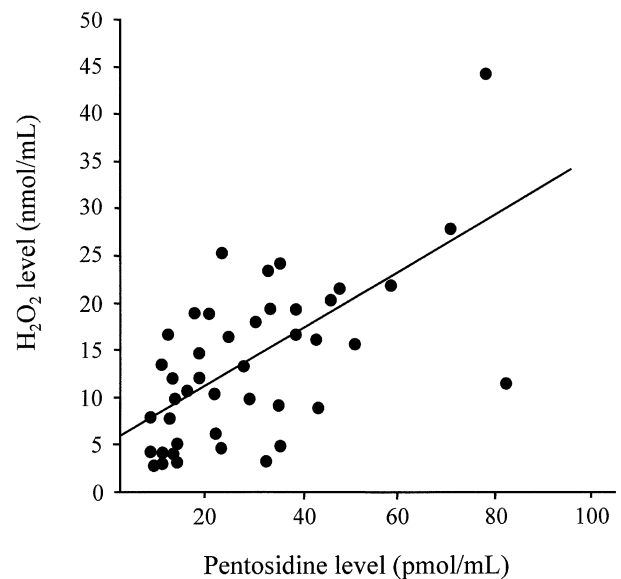

Figure 4. Correlations between intravitreal pentosidine and hydrogen peroxide (H_2O_2) in the groups with diabetic retinopathy. H_2O_2 (nmol/mL) = 0.255 × (pentosidine; pmol/ml) + 5.243 (r = 0.58, P < .01, Pearson correlation coefficients). The line indicates the linear regression equation.

pentosidine and VEGF, pentosidine and H_2O_2, and H_2O_2 and VEGF in patients with DM (r = 0.62, 0.58, 0.65; P < .01, respectively), especially in the VH group (r = 0.81, 0.63, 0.81; P < .01, respectively).

The correlation coefficients and P value were r = 0.59 and P < .01 for total TGF-β_2 and MCP-1 in the DR group (Figure 6). The breakdown by retinal complications resulted in a correlation coefficient of r = 0.87 and P < .05 in the group with TRD and r = 0.62 and P < .01 in the group with TRD plus VH. In addition, VEGF and mature TGF-β_2 were significantly correlated only in the group with TRD plus VH (r = 0.68, P < .01).

In the control eyes, no significant correlation was found between pentosidine, any cytokines, and H_2O_2.

Discussion

There are three possible mechanisms to explain the significantly high vitreous pentosidine levels in the DR groups. (1) In persistent hyperglycemia, reducing sugar including glucose enters the vitreous and causes a glycation reaction with vitreous collagen, which has a low metabolic turnover rate, and then the late-stage reactions proceed through the oxidation in the vitreous body. (2) Augmented perme-

ability after the collapse of the blood retinal barrier[18] causes serum proteins to enter the vitreous and undergo glycation reaction, or causes AGE-peptides, AGE-proteins,[19] and pentosidine[20] in the serum to flow into and accumulate in the vitreous. (3) Serum proteins and pentosidine are physically dispersed as a result of VH. However, the third mechanism is unlikely because the results in the present study indicated that pentosidine levels in the vitreous of DR without VH (the TRD and the DME groups) were, like the VH and the VH plus TRD groups, higher than in the control group, and that the serum HbA1c level, which positively correlates with the serum pentosidine level,[5] did not correlate with the vitreous pentosidine levels despite the presence of VH. VH resulting from vascular collapse is the only factor that explains the significantly high pentosidine levels in the vitreous of the DR groups.

The levels of pentosidine and VEGF in the DR groups were significantly higher than in the control group and there was a positive correlation between them. This would indicate that pentosidine, through AGE receptors, is involved in the enhancement of VEGF secretion from macrophages that infiltrate into the propagating cells and the vitreous body. In this study, the degree of DR progression and the concentration gradient of pentosidine and VEGF
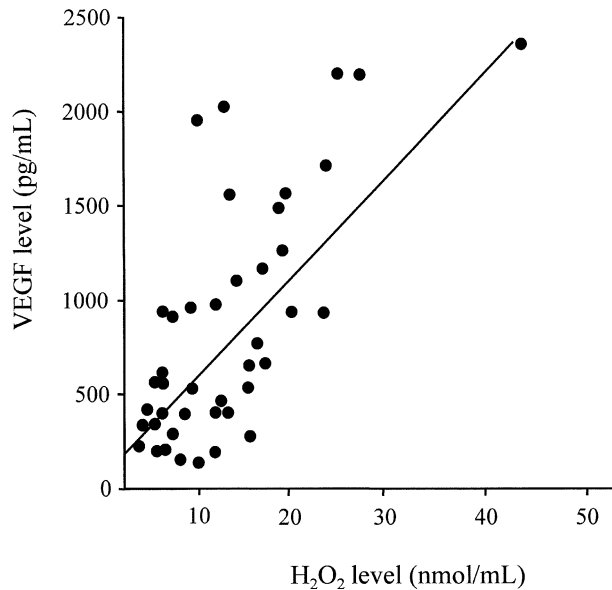
Figure 5. Correlations between intravitreal vascular endothelial growth factor (VEGF) and hydrogen peroxide (H_2O_2) in the groups with diabetic retinopathy. VEGF (pg/mL) = 52.40 × (H_2O_2; nmol/mL) + 171.29 ($r = 0.65$, $P < .01$, Pearson correlation coefficients). The line indicates the linear regression equation.

Figure 6. Correlations between intravitreal total transforming growth factor (TGF)-β_2 and monocyte chemotactic protein (MCP)-1 in the groups with diabetic retinopathy. Total TGF-β_2 (pg/mL) = 0.705 × (MCP-1; pg/mL) + 1386.7 ($r = 0.59$, $P < .01$, Pearson correlation coefficients). The line indicates the linear regression equation.

levels did not correlate in the investigations done based on the preoperative type of DR. This may suggest that retinal ischemia caused by TRD, which leads to the progression of DR, results in VEGF secretion without AGE involvement.

MCP-1 has a chemotactic action on monocytes, macrophages, and T-lymphocytes, and monocytes and macrophages activated by MCP-1 produce TGF-β, tumor necrosis factor-α, and other factors. Because MCP-1 levels were significantly higher ($P < .05$) in the TRD plus VH group compared with the VH and the DME groups in the examination based on the preoperative type of DR, we hypothesized that there may be a direct relation between MCP-1 levels and the degree of DR progression. Further studies are required to determine the mechanism. In this study, no correlation was observed among the MCP-1, pentosidine, and VEGF levels. However, because pentosidine and the VEGF levels were relatively high in the DME group, which showed only a weak proliferative change, and because VEGF expression in the retina was enhanced even before the onset of DR,[18] we theorized that VEGF, which also enhances macrophage chemotactic activity, causes monocytes and macrophages to migrate into the vitreous like MCP-1 at the early stage of DR,[15] and the macro-
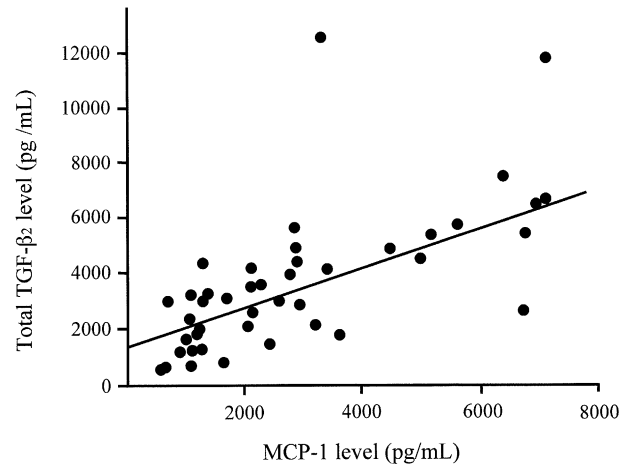
phages secrete various cytokines through AGE receptors.

As a result of the examination based on the preoperative type of DR in this study, a significant ($r = 0.68$, $P < .01$) relation between VEGF and mature TGF-β_2 levels in the vitreous was observed in the TRD plus VH group. The correlation between TGF-β_2 and VEGF expression levels in the TRD plus VH group may exist because of local production of both cytokines by retinal cells.[14] The significant relation between VEGF and mature TGF-β_2 levels observed in the TRD plus VH group is thought to result from repeated cycles of their exposure to each other.

In this study, the average levels of total TGF-β_2 and MCP-1 showed similar fluctuations by postoperative DR types. In particular, a strong positive relation ($r = 0.87$, $P < .05$; $r = 0.62$, $P < .01$) was observed in the groups with TRD. It is highly possible that TGF-β_2 secretion from macrophages and lymphatic cells that infiltrate into the vitreous because of MCP-1, retinal constituent cells, and vitreous cells are enhanced in the TRD group with strong proliferative changes. Lu et al[21] reported that injection of AGEs into the vitreous enhances VEGF expression in retinal pigment epithelium (RPE) cells in the presence of active oxygen like hydroxy radicals. Kuroki et al,[22] who reported the relation between glycoxidation and VEGF, demonstrated VEGF production from cultured RPE cells through H_2O_2 and superoxides. Superoxides generated through AGE-forma-

tion processes are thought to form H_2O_2 through the dismutation reaction, which leads to further accumulation of pentosidine. The present study showed that pentosidine, an AGE, H_2O_2, a form of active oxygen, and cytokines, including VEGF, TGF-β_2, and MCP-1, fluctuate interactively in the DR vitreous, depending on the type of DR.

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