

Amelioration of experimental autoimmune uveoretinitis by inhibition of glyceraldehyde-derived advanced glycation end-product formation

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ABSTRACT

AGEs are permanently modified macromolecule derivatives that form through nonenzymatic glycation of amino groups of proteins. Glycer-AGEs are highly toxic and play an important role in the pathogenesis of chronic inflammatory diseases. However, the contribution of glycer-AGEs to the pathogenesis of uveitis is unclear. In this study, we measured serum levels of glycer-AGEs in 100 patients with endogenous uveitis (22 with HLA-B27-associated uveitis, 20 with VKH disease, 14 with Behçet's disease, and 44 with sarcoidosis) and 33 healthy volunteers. We then examined the effect of the AGE inhibitor in a mouse model of human endogenous uveitis (EAU) by continuous oral administration of pyridoxamine at 200 or 400 mg/kg/day. Regardless of the etiology, serum glycer-AGE levels were significantly higher in patients with uveitis than in healthy subjects. Treatment with 400 mg/kg pyridoxamine significantly reduced the clinical and histological severity of EAU and was accompanied by a significant decrease in serum and retinal glycer-AGE levels and suppression of translocation of NF- κ B p65 into the nucleus of retinal cells. Serum glycer-AGE levels may therefore serve as a biomarker of human uveitis, as well as systemic inflammation, and may contribute to the progression of uveitis, including diabetic iritis, via the activation of NF- κ B. *J. Leukoc. Biol.* 96: 1077–1085; 2014.

Abbreviations: AGE=advanced glycation end-product, AP=alkaline phosphatase, CPM=counts per minute, DDW=double-distilled water, DLN=draining lymph node, EAU=experimental autoimmune uveoretinitis, glycer-AGE=glyceraldehyde-derived advanced glycation end-product, INL=inner nuclear layer, ONL=outer nuclear layer, PTX=*Bordetella pertussis* toxin, RAGE=receptor for advanced glycation end-product, VKH=Vogt-Koyanagi-Harada

Introduction

Uveitis is a sight-threatening ocular disease with various origins. Although autoimmune disorders have been implicated in the pathogenesis of endogenous uveitis, the precise cause remains elusive. An animal model of human endogenous uveitis, EAU, can be induced by immunization of mice or rats with high susceptibility to antigens derived from retinal proteins [1]. The EAU model has been widely used in the investigation of the pathogenesis of uveitis and for the evaluation of therapeutic effects of a variety of agents on uveitis.

AGEs are complex and heterogeneous compounds that are formed through nonenzymatic glycation of amino groups in proteins, lipids, and nucleic acids [2, 3]. This nonenzymatic reaction, also known as the Maillard reaction, consists of a series of reactions that sequentially form Schiff bases, ketoamine Amadori products, and finally, AGEs. AGEs accumulate with aging and have been implicated in various chronic inflammatory conditions, including rheumatoid arthritis and diabetes mellitus, and particularly in diabetic complications, such as retinopathy, neuropathy, and nephropathy [4–6]. The binding of an AGE to its receptor (RAGE) can induce proinflammatory cytokine responses via activation of p21^{ras}/MAPK and subsequent activation of transcription factor NF- κ B, eventually resulting in morphological and functional damage [7–11].

Six types of AGEs, designated as AGE-1 to AGE-6, have been identified from the serum of patients with diabetes mellitus [12–15]. Of these, glycer-AGEs (type AGE-2) are reportedly toxic and involved in the pathogenesis of various chronic diseases [16, 17]. In addition to their contribution to the patho-

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genesis of diabetes mellitus [18, 19], glycer-AGEs may play certain pathological roles in Alzheimer's disease, enhance the proliferation and activation of hepatic stellate cells, increase permeability of brain microvascular endothelial cells, and even enhance the malignancy of cancer [20–25]. However, the precise mechanism through which AGE formation is up-regulated under inflammatory conditions remains unclear.

Pyridoxamine, one of the derivatives of vitamin B6, can inhibit the conversion of Amadori intermediates to AGEs, suggesting its potential as a therapeutic agent for the treatment of diseases associated with AGEs [26, 27]. To date, pyridoxamine has been reported to retard the development of diabetic nephropathy and prevent arterial stiffening of diabetes in a streptozotocin-treated rat model for type 1 diabetes mellitus through the inhibition of AGE formation [28, 29]. It also improves the urinary albumin:creatinine ratio through its anti-AGE and antioxidant effects in the kidneys of KK-A^y/Ta mice [30] and inhibits AGE formation in the diabetic lens and the development of retinopathy in experimental animal models [31, 32].

Although evidence supporting the contribution of glycer-AGEs in the pathogenesis of a variety of diseases is increasing, the involvement of glycer-AGEs in uveitis has rarely been investigated. Uveitis is also characterized as a form of systemic inflammatory disease and occurs as acute anterior uveitis in uncontrolled diabetic patients in a form known clinically as “diabetic iritis” or “diabetic uveitis” of unknown cause. Thus, glycer-AGEs may possibly contribute to the pathogenesis of uveitis, and the inhibition of glycer-AGE formation may show suppressive effects on uveitis. We examined this hypothesis by measuring the serum glycer-AGE levels of patients with HLA-B27-associated uveitis, VKH disease, Behçet's disease, and sarcoidosis, which are considered to be the most common etiologies of endogenous uveitis in secondary and tertiary eye centers in Japan [33–35]. We then investigated whether inhibition of glycer-AGE formation by pyridoxamine can ameliorate uveitis in the EAU model in mice.

MATERIALS AND METHODS

Patients

In total, 100 consecutive patients diagnosed with uveitis, who visited the Uveitis Clinic of Hokkaido University Hospital between 2000 and 2004, were enrolled in the study. These included 22 patients with HLA-B27-associated uveitis, 44 with sarcoidosis, 20 with VKH disease in the convalescent stage, and 14 with Behçet's disease (Table 1). An additional 33 healthy vol-

unteers were enrolled as controls. The absence of a statistical difference in ages among any of the patient groups and the healthy controls was confirmed by using Kruskal-Wallis analysis. No cases of diabetes were reported in the groups of study patients and healthy volunteers. After informed consent, in accordance with the tenets of the Declaration of Helsinki, was obtained from each patient and control subject, sera were collected for the measurement of glycer-AGEs.

Experimental animals

Female B10.BR (H-2^k) mice (6 weeks old) were obtained (Japan SLC, Hamamatsu, Japan). All studies were conducted in compliance with the Association for Research in Vision and Ophthalmology Statement for the Use of Animals in Ophthalmic and Vision Research and were approved by the Ethics Review Committee for Animal Experimentation of Hokkaido University.

Reagents

The K2 peptide (ADKDVVLTSSRTGGV), corresponding to the amino acid sequence 201–216 of the bovine interphotoreceptor retinoid-binding protein and synthesized by Sigma-Genosys Japan (Ishikari, Hokkaido, Japan), was used as the immunodominant retinal autoantigen of EAU in the H-2^k mice [36]. Purified PTX was purchased from Sigma-Aldrich (St. Louis, MO, USA). CFA and *Mycobacterium tuberculosis* strain H37Ra were purchased from Difco (Detroit, MI, USA). Pyridoxamine was kindly provided by Kowa, Fuji Research Laboratories (Fuji-sh, Shizuoka, Japan).

Measurement of serum levels of glycer-AGEs

Serum levels of glycer-AGEs were measured with a competitive ELISA, as reported previously [13]. For serial measurement of mouse serum glycer-AGE levels during the experiment, a slight incision was made in the tail following anesthesia with ether, sterilization was achieved with cotton soaked in 70% alcohol, and blood was collected using a pipette. The blood was transferred into microtubes and centrifuged for 20 min at 4°C, 13,850 g, and measurements were made using the supernatants.

Induction of EAU and administration of pyridoxamine

EAU was induced in the H-2^k mice by s.c. injection of 100 nmol K2 peptide, which was emulsified in CFA containing 2.5 mg/ml *M. tuberculosis* strain H37Ra. Injections were made in the upper back and flanks and followed concurrently by the i.p. injection of 0.1 µg PTX in 100 µl PBS. Pyridoxamine was supplied in aluminum foil-covered drinking bottles as a result of its photosensitivity. Five mice were caged with each drinking bottle. The dosage of added pyridoxamine was calculated based on consumption during the preliminary experiment.

The mice were treated with oral pyridoxamine (200 mg/kg/day, low-dose, or 400 mg/kg/day, high-dose) or DDW as the control, starting at the time of EAU induction, to examine the suppressive effect of pyridoxamine on EAU. After determination of the optimal dose of pyridoxamine, the mice were divided into three groups for further experiments: (1) naive mice treated with oral administration of an optimal dose of pyridoxamine, (2) EAU-mice treated with an optimal dose of pyridoxamine, and (3) EAU mice given DDW as controls. There were no observable effects of using DDW daily on the health of the mice during the study.

Evaluation of EAU

Starting 7 days after immunization, the uveoretinal inflammation was assessed by funduscopy every 3–4 days. The severity of retinal inflammation was graded on a five-point scale, as described previously [37, 38]. Clinical scoring was based on vessel dilatation; number of white focal or liner chorioretinal lesions along the vessels; and extent of exudate of retinal vessels, hemorrhage, and retinal detachment.

On Day 21 after induction of EAU, the mice were euthanized with an overdose of anesthetic. The eyes were enucleated and fixed in 4% phos-

TABLE 1. Characteristics of Patients

Diseases	Number of cases	Gender		Age (year) mean ± SEM
		Female	Male	
HLA-B27	22	8	14	41.8 ± 3.0
Sarcoidosis	44	31	13	43.7 ± 2.8
VKH	20	13	7	46.4 ± 2.9
Behçet	14	8	6	38.6 ± 3.0
Healthy	33	19	14	37.1 ± 2.0

phate-buffered glutaraldehyde for 1 h before 4% paraformaldehyde for preparation of paraffin sections. Fixed tissues were stained with H&E. Histologic severity was graded on a scale of zero to four, as described previously [1]. Briefly, the histological score was based on the degree of cell infiltration, vasculitis, granuloma formation, photoreceptor cell damage in the retina, and retinal detachment in the eye. The higher score of the two eyes was adopted as the severity in each mouse.

Immunohistochemistry

On Day 10 after induction of EAU, the mice were euthanized with an overdose of anesthetic for immunohistochemistry. Fixation by intracardiac perfusion of 4% paraformaldehyde in PBS, followed by enucleation, was performed to examine the glycer-AGE accumulation in mouse retinas and the translocation of NF- κ Bp65. The eyes were immersed in the same fixative for 24 h, followed by embedment and sectioning.

The accumulation of glycer-AGEs in the mouse retinas was examined in serial paraffin sections of the retinas. These were dewaxed in xylene and rehydrated in ethanol of descending concentrations, followed by microwave-based antigen retrieval using Dako Target Retrieval Solution (Dako North America, Carpinteria, CA, USA) for 15 min, inactivation of endogenous peroxidase by incubation with 3% H₂O₂ for 10 min, and incubation with Dako Protein Block (Dako North America) for 2 h. The sections were then incubated with a rabbit anti-glycer-AGE antibody [13] (1:1000 dilution) overnight at 4°C, followed by incubation with a biotinylated secondary antibody (1:1000 dilution; Jackson ImmunoResearch Laboratories, West Grove, PA, USA) at room temperature for 1 h. The sections were then incubated with a Vectastain Avidin Biotinylated Enzyme Complex (ABC)-AP Kit (Vector Laboratories, Burlingame, CA, USA) for 30 min and in AP substrate solution 5-bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium (BCIP/NBT) AP Substrate Kit IV; Vector Laboratories) for 1 h, according to the manufacturer's protocols, to develop blue reaction products. The sections were then dehydrated with ethanol and xylene, mounted with mounting medium (MP500; Matsunami, Osaka, Japan), and coverslipped. All of the sections were examined by microscopy (Biorevo/Keyence, Osaka, Japan). One area that included the whole sensory retina was photographed randomly, and the brightness value of glycer-AGEs, which stained blue, was calculated using the analyzing unit installed in the microscope (BZII analyzer; Keyence). As stronger immunoreactivity shows darker blue, a low brightness value means large amounts of glycer-AGE. The results were made easier to understand by expressing them as the ratio of the mean brightness value of retinas of naive mice given pyridoxamine. Four samples were enrolled from each group. All immunostaining procedures included negative controls to confirm the specificity of the immunostaining, using PBS instead of primary antibodies.

The translocation of NF- κ B p65 was examined by dewaxing slides in xylene and then rehydrating in ethanol of descending concentrations. Microwave-based antigen retrieval was performed in 100 mM citrate buffer (pH 6.0), followed by incubation in 0.1% BSA for 30 min. Sections were incubated with rabbit anti-NF- κ B p65 antibody (1:50 dilution; C22B4; Cell Signaling Technology, Danvers, MA, USA). Binding of primary antibodies was localized with Alexa Fluor 546 goat anti-rabbit secondary antibody (1:100 dilution; Life Technologies, Carlsbad, CA, USA). Nuclei were stained with YO-PRO-1 (1:1000 dilution; Life Technologies) for 20 min. Finally, sections were mounted with Vectashield Mounting Medium (Vector Laboratories) and coverslipped. Photos were taken within the internal granular layer of each retinal sample with a microscope system (Biorevo/Keyence).

Determination of immunologic responses

Antigen-specific T cell proliferation was assayed in B10.BR mice immunized with K2 and treated with pyridoxamine or DDW ($n=3$, respectively). Primed lymphocytes were obtained from DLNs (axillary, cervical, and inguinal), 10 days after immunization. T cell-enriched fractions were prepared by passing the dispersed cells from the DLNs of these K2-primed mice over nylon wool columns. Nylon wool nonadherent cells (5×10^5 /well) were cultured with mitomycin C-treated splenocytes as APCs (1×10^5 /

well) and various concentrations of K2 peptide in a 96-well flat-bottomed microtiter plate for 72 h at 37°C. The cells were then pulse labeled with [³H]-thymidine (Perkin Elmer Japan, Tokyo, Japan) and incubated for 16 h. Incorporation of [³H]-thymidine was quantified with a direct β -counter (Packard, Meriden, CT, USA), and data were presented as mean CPM minus the background (medium alone; Δ CPM), as described previously [38]. Background CPM was from wells of medium alone with [³H]-thymidine but not with cells.

Quantification of cytokines

The concentrations of Th1/Th2/Th17 and inflammatory cytokines (including IFN- γ , TNF- α , IL-1 α , IL-2, IL-4, IL-5, IL-6, IL-10, and IL-17) and a measurement of GM-CSF in culture supernatant of the antigen-specific T cell response were quantified with the mouse Th1/Th2 10-plex FlowCytomix Multiplex (Bender MedSystems GmbH, Vienna, Austria), according to the manufacturer's protocol with a flow cytometer.

Statistical analysis

Results were presented as mean \pm SD. Statistical analysis was performed using the Kruskal-Wallis test for comparing three or more groups, and the Mann-Whitney *U*-test for comparing two groups, respectively. $P < 0.05$ was considered statistically significant.

RESULTS

Serum levels of glycer-AGE were significantly higher in patients with endogenous uveitis

The involvement of glycer-AGEs in the pathology of human endogenous uveitis was examined by ELISA measurements of serum glycer-AGE levels in patients with HLA-B27-associated uveitis ($n=22$), VKH disease ($n=20$), Behçet's disease ($n=14$), and sarcoidosis ($n=44$) and then comparing these with the levels in healthy controls ($n=33$). Their clinical characteristics are summarized in Table 1. The mean age of the different patient groups was not significantly different. Serum glycer-AGE levels in patients with HLA-B27-associated uveitis, sarcoidosis, VKH disease, and Behçet's disease were 7.38 ± 0.57 , 7.27 ± 1.19 , 6.74 ± 0.24 , and 6.45 ± 0.97 U/ml, respectively. Serum glycer-AGE levels were significantly higher for each etiology of uveitis than in the healthy controls (4.16 ± 0.26 U/ml, $P < 0.01$ for Behçet's disease; $P < 0.001$ for the rest; **Fig. 1**).

Amelioration of EAU by oral administration of pyridoxamine

The suppressive effects of pyridoxamine on uveoretinitis were examined in EAU-induced mice treated with oral pyridoxamine at a dose of 200 mg/kg/day (low dose) or 400 mg/kg/day (high dose) or only DDW throughout the experimental period. Uveoretinitis was clinically diagnosable, ~10 days after immunization, and peaked on Day 21 in all EAU groups. The clinical score of EAU was lower in mice treated with 400 mg/kg/day pyridoxamine than in the mice of the other two groups at all examination points, especially at Day 19 and Day 21 ($n=10$ for each group, $P < 0.05$; **Fig. 2A**). The mice treated with 200 mg/kg/day pyridoxamine or DDW showed no significant differences in EAU clinical scores throughout the experimental period.

Histological evaluation of EAU was conducted by enucleating eyes 21 days after EAU induction and performing his-

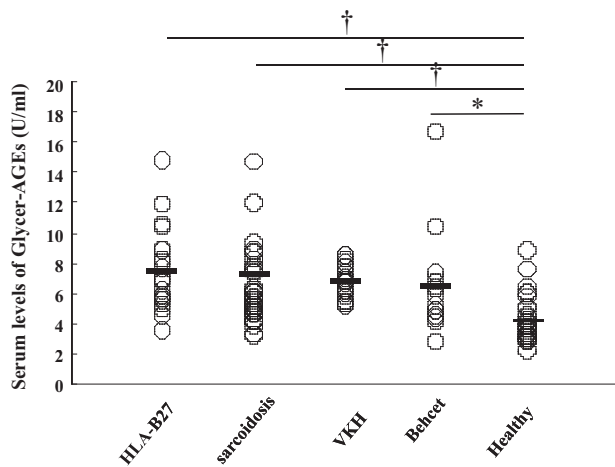


Figure 1. Serum glycer-AGE levels in patients with endogenous uveitis and healthy controls. The average serum glycer-AGE levels were significantly higher in patients with HLA-B27-associated uveitis ($n=22$), VKH disease ($n=20$), Behçet disease ($n=14$), and sarcoidosis ($n=44$) than in healthy controls ($n=33$). Horizontal bars in the figure represent the average of each group. Significance was determined by Kruskal-Wallis test, $*P < 0.05$; $†P < 0.01$.

topathological examinations. Representative H&E histology of EAU mice treated with 200 mg/kg/day pyridoxamine, 400 mg/kg/day pyridoxamine, or DDW is shown in Fig. 2. The EAU mice treated with only DDW (Fig. 2B) showed inflammatory cells in the retina, vitreous, and choroids, with retinal-fold and hemorrhagic lesions. The mice treated with 200 mg/kg/day pyridoxamine showed similar inflammatory lesions in the retinas (Fig. 2C), whereas the mice treated with 400 mg/kg/day pyridoxamine showed nearly normal

retinas (Fig. 2D). Consistent with the results of clinical severity, the histological severity of EAU was markedly milder in EAU mice treated with 400 mg/kg/day pyridoxamine ($n=20$; histologic score: 0.89 ± 0.17) than in those treated with DDW ($n=20$; histologic score: 1.82 ± 0.28 , $P < 0.05$; Fig. 2E). No significant difference in histologic score between mice treated with 200 mg/kg/day pyridoxamine ($n=16$; histologic score: 1.56 ± 0.25) and those treated with DDW was observed.

Decrease in serum glycer-AGE levels in response to oral pyridoxamine administration

The suppressive effect of pyridoxamine on serum glycer-AGEs was examined by ELISA measurements of serum glycer-AGE levels at Days 0, 10, and 21 after immunization. At Day 0, no significant difference in serum glycer-AGE levels could be seen among the three groups (naïve mice given 400 mg/kg/day pyridoxamine: 13.6 ± 0.7 U/ml; EAU mice treated with high-dose pyridoxamine: 13.0 ± 0.6 U/ml; EAU mice treated with DDW: 13.1 ± 0.5 U/ml). Serum glycer-AGE levels of naïve mice given oral pyridoxamine remained almost unchanged throughout the experiment period (14.8 ± 1.5 U/ml at Day 10 and 14.0 ± 0.4 U/ml at Day 21, respectively). In contrast, serum glycer-AGE levels in EAU mice treated with DDW were elevated significantly after immunization and remained at a high level, whereas EAU mice treated with high-dose pyridoxamine showed significantly lower serum glycer-AGE levels than EAU mice treated with DDW. These differences were especially apparent at Day 10 (31.9 ± 3.9 U/ml and 50.3 ± 7.0 U/ml, respectively; $P < 0.05$) and Day 21 (36.6 ± 5.4 U/ml and 56.3 ± 3.0 U/ml, respectively; $n=5-10$ for each group; $P < 0.05$; Fig. 3).

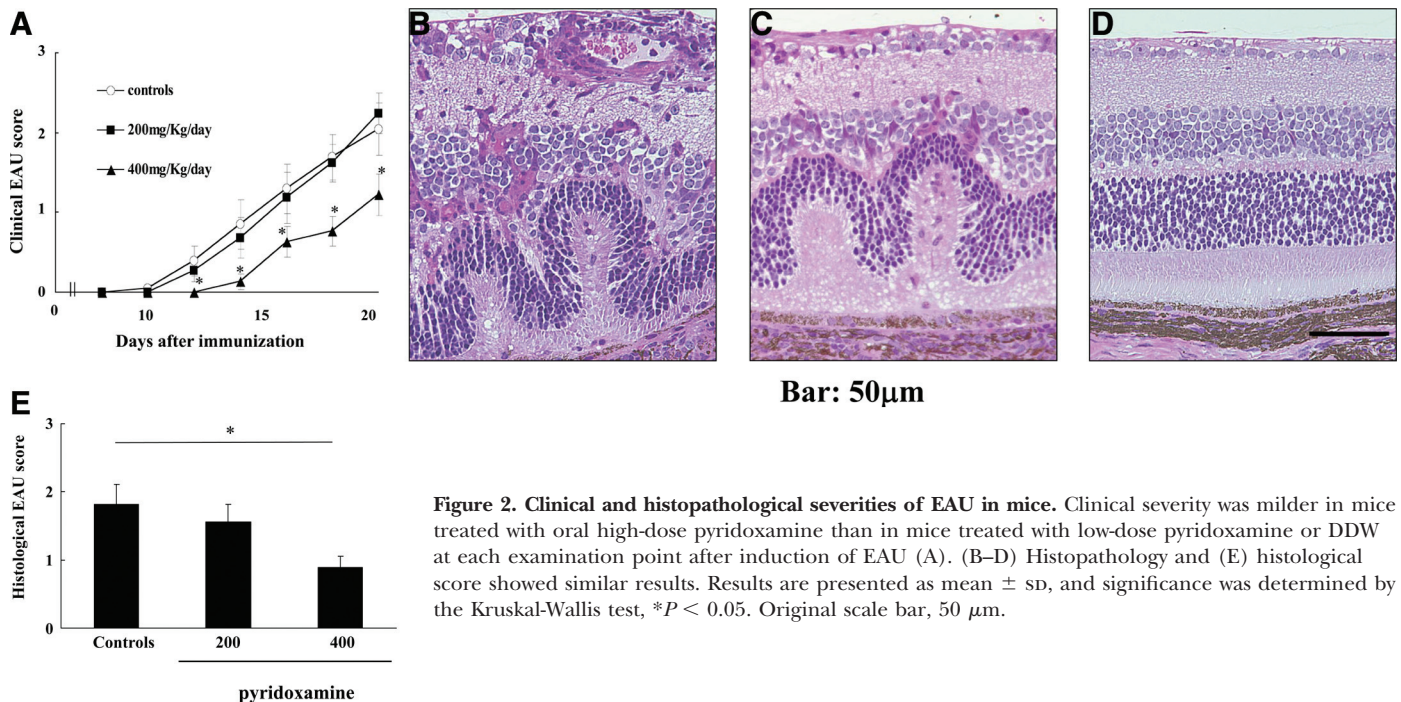


Figure 2. Clinical and histopathological severities of EAU in mice. Clinical severity was milder in mice treated with oral high-dose pyridoxamine than in mice treated with low-dose pyridoxamine or DDW at each examination point after induction of EAU (A). (B–D) Histopathology and (E) histological score showed similar results. Results are presented as mean \pm SD, and significance was determined by the Kruskal-Wallis test, $*P < 0.05$. Original scale bar, 50 μ m.

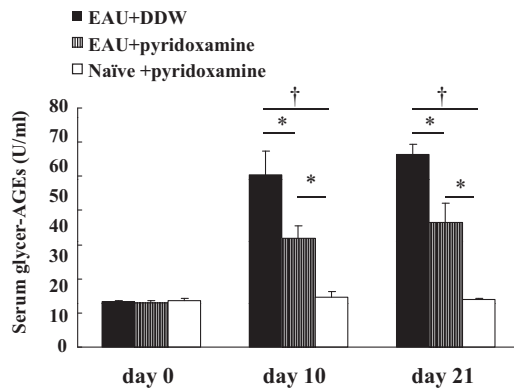


Figure 3. Serum levels of glycer-AGE in mice. Sera of mice were obtained at Days 0, 10, and 21 after immunization to quantify glycer-AGE concentrations with ELISA. At Days 10 and 21, serum levels of glycer-AGE were significantly lower in EAU mice administered oral pyridoxamine than in untreated EAU mice administered DDW. Results are presented as mean \pm sd, and significance was determined by Kruskal-Wallis test, $*P < 0.05$; $\dagger P < 0.01$.

Localization and reduction of glycer-AGE deposition in EAU retinas following oral pyridoxamine administration

The immunolocalization of glycer-AGEs in EAU retinas was examined in eyes obtained 10 days after the immunization. Immunohistochemical staining was performed with the antibody against glycer-AGEs and gave a blue reaction product. Glycer-AGE deposition was identified almost throughout the whole retina of EAU mice given only DDW, including the inner plexiform layers and the cytoplasm of the INL and ONL of the retina (Fig. 4A), whereas the deposition of glycer-AGE was notably weaker in the retinas of EAU mice treated with pyridoxamine (Fig. 4B). Almost no glycer-AGE deposition was identified in naive mice given 400 mg/kg/day pyridoxamine (Fig. 4C).

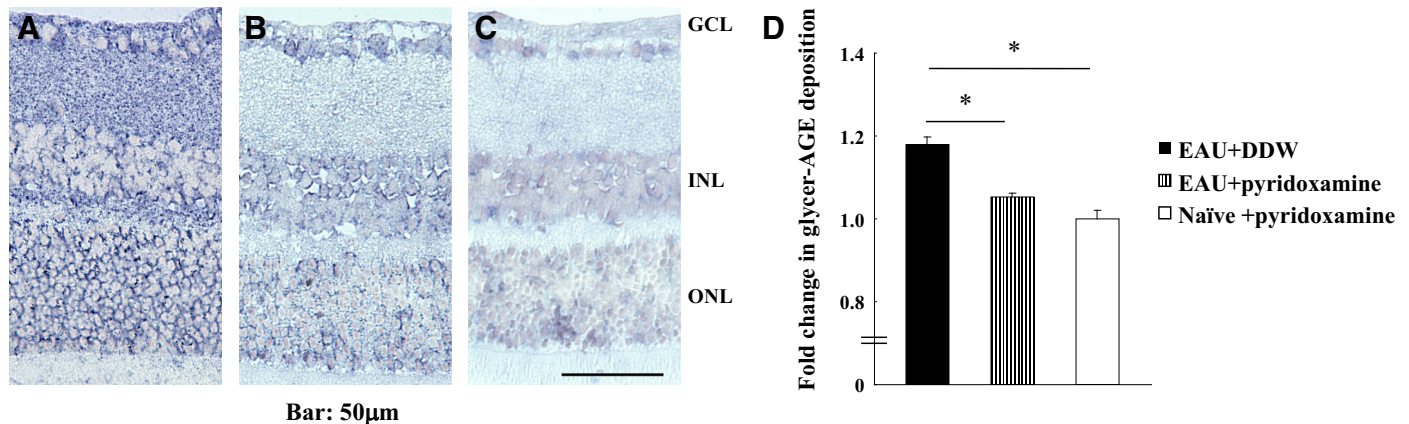


Figure 4. Localization and deposition of glycer-AGE in EAU retinas. The immunoreactivity of glycer-AGE (blue) in retinas of EAU mice given DDW (A) was increased significantly than that of naive mice given pyridoxamine as negative controls (C). It was decreased markedly by treatment with oral pyridoxamine (B). Results of quantification of glycer-AGE immunoreactivity express ratio to mean glycer-AGE deposition in retinas of naive mice given pyridoxamine and are presented as mean \pm sd. (D) Significance was determined by Kruskal-Wallis test, $*P < 0.05$. GCL, Ganglion cell layer. Original scale bar, 50 μ m.

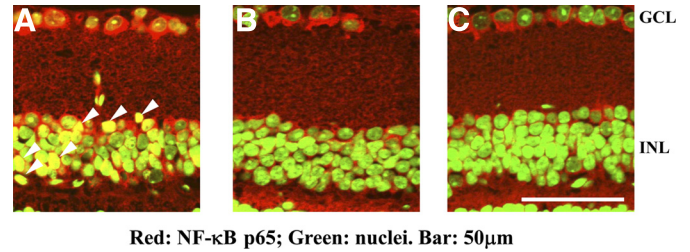


Figure 5. Translocation of NF- κ B p65 in EAU retinas. YO-PRO-1 nuclear staining (green) and immunodetection of NF- κ B p65 (red) in the retinas of EAU mice administered with DDW (A) or oral pyridoxamine (B) and naive mice given pyridoxamine (C). The translocation of NF- κ B into nuclei (indicated by white arrowheads) was induced by immunization (A); however, this translocation was markedly suppressed by administration of pyridoxamine (B). No NF- κ B translocation was observed in naive mice given pyridoxamine (C). Original scale bar, 50 μ m.

The amount of glycer-AGE deposition in retinas was quantified by calculating its brightness. The observations (Fig. 4A–C) were confirmed by the brightness calculations (Fig. 4D).

Inhibition of the translocation of NF- κ B into the nucleus in EAU retinas

The inhibitory effect of pyridoxamine on translocation of NF- κ B in the retina was examined in eyes obtained 10 days after the immunization. Immunohistochemical staining was performed with the antibody against NF- κ B subunit p65. Translocation of NF- κ B into the nucleus was confirmed, especially in the INLs of the retinas of EAU mice given only DDW without pyridoxamine. In contrast, almost no staining was detected in the retinas of the naive or EAU mice treated with pyridoxamine (Fig. 5).

Pyridoxamine showed little influence on the priming of antigen-specific T cell proliferation but reduced Th1/Th17-mediated cytokine production in vitro

In EAU, the Th1 and Th17 cells mediate inflammatory responses. These T cells show vigorous in vitro proliferation upon stimulation with immunized peptides presented by the APCs. We examined the mechanism underlying the suppressive effect of pyridoxamine by analyzing the proliferative responses to K2 peptides in T cells from pyridoxamine-treated and DDW-given EAU mice. Responder T cells were obtained from the DLN of EAU mice at Day 10 after K2 immunization and cultured with K2 and APCs, as described in Materials and Methods. No significant differences were observed in the antigen-specific T cell proliferation between pyridoxamine-treated and untreated EAU mice (**Fig. 6A**). However, Th1- and Th17-mediated cytokine levels in the culture supernatant, in particular, those of IL-2, IFN- γ , and IL-17, were reduced when treated with pyridoxamine. The IL-6 level was also reduced. By contrast, the level of the Th2-type cytokine IL-4 was not reduced by treatment with pyridoxamine (**Fig. 6B–H**). IL-1 α , IL-5, and IL-10 levels were under the detection limits of the ELISA. These findings suggest that pyridoxamine does not down-regulate the T cell proliferative response, whereas it does suppress the Th1/Th17 responses of EAU.

DISCUSSION

The contribution of AGEs to pathogenesis has been investigated widely, especially regarding involvement in diabetic complications [39]. The contribution of AGEs to endogenous uveitis, an ocular autoimmune disorder, has yet to be established. The present study demonstrates, for the first time, extraordinarily high levels of glycer-AGEs in the serum of patients with endogenous uveitis, even though these patients did not suffer from diabetes. We also demonstrated the prophylactic effects of pyridoxamine, which inhibits glycer-AGE formation, as a treatment for uveitis using an EAU model in mice. This response to pyridoxamine suggests that glycer-AGEs are involved in the pathogenesis of uveitis.

Mouse serum glycer-AGE levels were elevated dramatically after immunization, even at Day 10, when only slight EAU symptoms could be clinically observed. Glycer-AGE accumulation also increased significantly in EAU retinas, 10 days after immunization. In agreement with the mouse-model findings, quantification of serum glycer-AGE levels showed that patients with various forms of endogenous uveitis had significantly higher glycer-AGE levels than healthy controls. These findings strongly implicate involvement of glycer-AGEs in the pathogenesis of endogenous uveitis in humans.

Pyridoxamine was reported to inhibit AGE deposition in the kidneys in an experimental animal model of chronic allograft nephropathy, in skin collagen, and in the retinas of diabetic

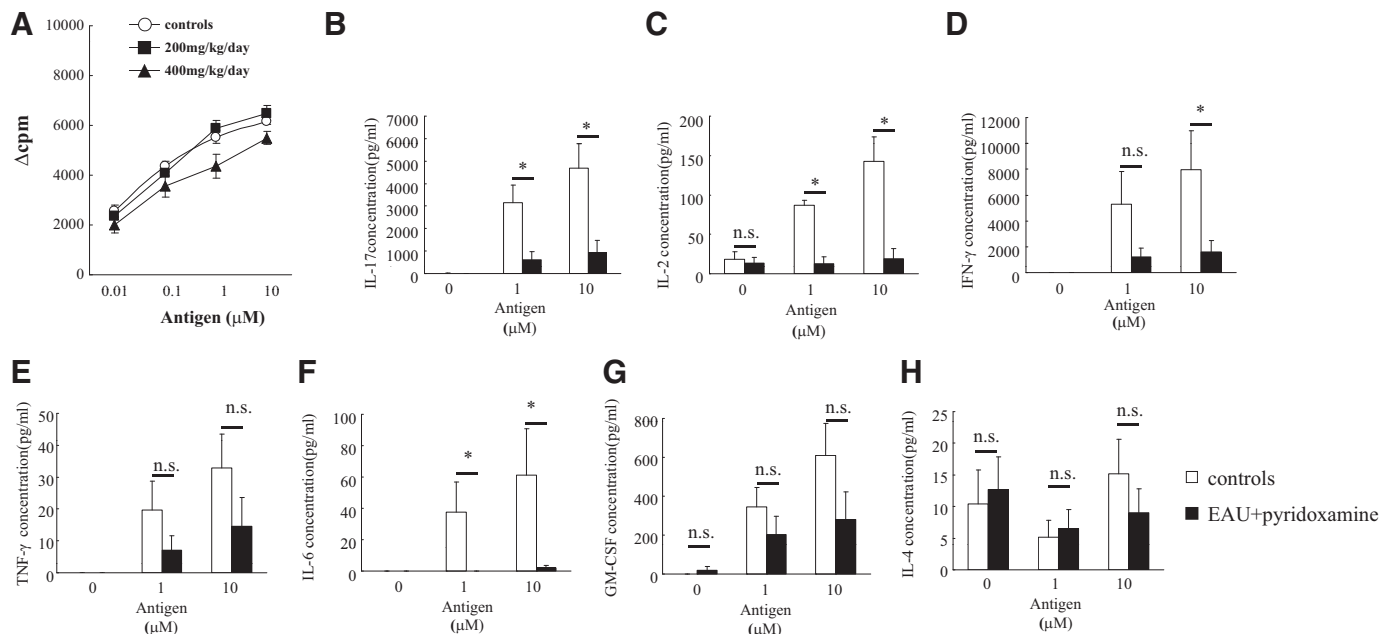


Figure 6. Antigen-specific T cell proliferation and cytokine in vitro. (A) T cells were obtained from the DLNs of B10.BR mice, 10 days after immunization, incubated with the indicated doses of antigen peptide K2 in the presence of APCs, and pulse-labeled with [3 H]-thymidine for the last 16 h. T cells from both pyridoxamine-treated and untreated groups showed similar vigorous responses ($n=3$ for each group, respectively). (B–H) Cytokine production by T cells in the culture supernatant was quantified. Th1 and Th17-mediated cytokines, in particular, those of IL-2, IFN- γ , and IL-17 were reduced in the treatment with pyridoxamine. However, IL-4, a Th2-mediated cytokine, was not decreased. Results are expressed as mean \pm SE. Significance was determined by Mann-Whitney U -test; $n=4$ for control group, and $n=5$ for pyridoxamine-treatment group, respectively; * $P < 0.05$.

animals [29, 32, 40, 41]. Consistent with those previous reports, pyridoxamine significantly decreased the serum glycer-AGE levels and reduced glycer-AGE deposition in EAU retinas in parallel with amelioration of EAU in mice, adding another layer of evidence for the involvement of glycer-AGEs in the pathogenesis of EAU.

The mouse EAU model of human endogenous uveitis is induced by the infiltration of pathogenic T cells and the subsequent influx of other leukocytes into the retina and the uveal tract [42, 43]. The immune and inflammatory reactions evoked and mediated by the accumulating leukocytes are mediated by NF- κ B, which plays a crucial role as it translocates from the cytoplasm into the nucleus. There, NF- κ B activates genes for various proinflammatory cytokines; thus, it has long been a therapeutic target. Several NF- κ B inhibitors show anti-inflammatory effects, by preventing phosphorylation of I κ B, which binds with NF- κ B and prevents it from entering the nucleus in the absence of stimuli [44], or by directly inhibiting translocation of NF- κ B into the nucleus [45, 46]. In this study, we confirmed translocation of NF- κ B into the nucleus, mainly in the INL of the retinas of EAU mice, in agreement with our previous reports [45, 46]. We have now demonstrated that this translocation is inhibited by pyridoxamine. Furthermore, it is possible that oral pyridoxamine also inhibits NF- κ B translocation in infiltrating inflammatory cells; this needs to be clarified with further examination. However, pyridoxamine did not suppress antigen-specific T cell-proliferative responses, which may be a reason that it partially suppressed EAU. Antigen-specific T cell proliferation is obtained as a sum of Th1/Th17 and Th2 responses. The cytokine assay showed that pyridoxamine suppressed Th1/Th17 responses but caused only a small Th2 response. As EAU is considered primarily a Th1/Th17-mediated inflammatory model of the eye, amelioration of EAU with pyridoxamine is rational.

However, some questions remain. Pyridoxamine suppressed the NF- κ B activation of resident retinal cells and also suppressed Th1/Th17 responses collected from DLNs at Day 10 after immunization. At Day 10, most of the primed T cells have not yet reached the eyes but still remain in the lymph nodes. Does oral pyridoxamine inhibit NF- κ B translocation of infiltrating inflammatory cells, as well as resident retinal cells in the eye? To clarify the association of NF- κ B suppression with pyridoxamine for resident retinal cells and infiltrating cells in the eye, NF- κ B translocation may be examined, according to time in future projects. Second, the possibility that the increase in glycer-AGE formation in patients with EAU and uveitis is a bystander effect secondary to inflammation cannot be excluded. The determination of whether glycer-AGE levels in sera or retinas are a result or one of the causes of ocular inflammatory disorders is not easy. Some reports have shown deposition of AGEs in retinas [47] and have indicated that AGE formation might induce and/or up-regulate inflammatory cytokines [24, 48, 49]. Glycer-AGEs bind to RAGEs and induce reactive oxygen species production through NADPH oxidase activation, resulting in NF- κ B translocation into the nucleus via activation of p21^{ras}/MAPK [48]. However, it is not well understood what triggers glycer-AGE formation under in-

flammatory conditions of the eye and vice versa. The current results show that EAU severity is in parallel with increases in glycer-AGE accumulation in the retinas and sera. The accumulation of glycer-AGEs may exacerbate EAU via binding with RAGEs and the subsequent serial activations of intracellular signal transduction and eventual up-regulation of inflammatory factors. This, in turn, leads to further accumulation of glycer-AGEs and more severe inflammation, as suggested in another disease [24], creating a perpetual cycle. Inhibition of glycer-AGE formation may therefore interrupt this cycle by decreasing both serum and local glycer-AGE levels, thereby ameliorating EAU.

We reported previously involvement of glycer-AGEs at the onset of disease in VKH patients in whom glycer-AGE levels decreased at the very acute phase of the disease and then rose when the patients recovered, following treatment with systemic corticosteroids [50]. This particularity may be a result of the characteristic pathogenesis of the immunological reaction against melanocytes in VKH disease [51, 52], which differs from HLA-B27-associated uveitis, Behçet's disease, and sarcoidosis. The present data that indicate proinflammatory, rather than autoimmune, effects of glycer-AGEs are also consistent with the clinical features of diabetic iritis, where acute uveitis occurs with no autoimmune T cells among poorly controlled diabetic patients. Further clinical studies are required to quantify serum glycer-AGE levels in diabetic iritis patients. However, this can be a bit difficult, as diabetic iritis may recover immediately, and poorly controlled diabetic patients will show high serum glycer-AGE levels as a result of hyperglycemia, even before and after the occurrence of diabetic uveitis.

From the clinical perspective, our experiments showing the effect of pyridoxamine indicate the possibility of prophylactic administration for patients with recurrent uveitis. In conclusion, glycer-AGE levels were extraordinarily higher in sera from patients with endogenous uveitis than in sera from healthy people. Glycer-AGE levels were elevated significantly in sera and retinas after induction of EAU in mice but decreased following glycer-AGE inhibition by pyridoxamine. NF- κ B translocation into the nucleus was inhibited in parallel with amelioration of EAU, both clinically and histopathologically. Glycation reactions between sugars and amino acids greatly contribute to endogenous uveitis in vivo, as seen in diabetic retinal complications, which are now considered to reflect chronic microinflammation. The targeting of glycer-AGEs as novel therapeutic candidates therefore appears to have much potential in the treatment of ocular endogenous inflammatory disorders.

AUTHORSHIP

N.K. designed the research and conducted experiments. Z.D. and N.K. wrote the paper. Z.D., D.I., M.T., M.S., N.E., K.I., A.L., and M.K. performed the experiments and/or analyzed data. D.I., N.K., M.K., R.A., J.F., S.K., K.M., K. Namba, and S.O. examined the patients, obtained informed consent, and collected clinical samples. M.T., K.I., A.K., K. Noda, S.I.Y., S.O., and S.I. provided important and intellectual input into

the research. All authors contributed to discussion of the results, followed by writing and reviewing the manuscript.

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DISCLOSURES

The authors declare no conflict of interest.

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