Changes in Interleukin 18 in the Retinas of Otsuka Long-Evans Tokushima Fatty Rats, a Model of Human Type 2 Diabetes

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Abstract: Recent findings have implicated the involvement of interferon- γ (IFN- γ), a part of the Th1 cytokine response, in the retinal inflammation of diabetic patients. In the present study, we investigate whether hyperglycemia relates to the expression of interleukin 18 (IL-18), and leads to the production of IFN- γ in the retinas of Otsuka Long-Evans Tokushima Fatty (OLETF) rats, a model of type 2 diabetes mellitus. Plasma blood glucose, triglyceride and cholesterol levels in 60-week-old OLETF rats, in which the development of diabetes mellitus was observed, were significantly higher than in 60-week-old Long-Evans Tokushima Otsuka (LETO) rats used as normal controls. The expression levels of genes that cause IL-18 activation (IL-18, IL-18 receptor and caspase-1) in OLETF rats were increased at 60 weeks of age, and the levels of IL-18 and IFN- γ in 60-week-old OLETF rat retinas were also higher than in 60-week-old LETO rats. Furthermore, IFN- γ levels increased with increasing IL-18 levels in the retinas of OLETF rats, and a close relationship was observed between the levels of IL-18 and HbA1c. The rapid increase in plasma glucose levels following the oral administration of glucose solution (3.0 g/kg) did not affect the IL-18 and IFN- γ levels in the retinas of LETO rats, whereas the levels in the retinas of OLETF rats increased significantly. In conclusion, the expression of IL-18 is increased in the retinas of OLETF rats, and chronic hyperglycemia may accelerate the release of IL-18 and IFN- γ from inflammatory cells in retinal blood vessel. It is possible that IFN- γ production *via* IL-18 in the retinas of 60-week-old OLETF rats is caused by hyperglycemia, and plays a role in the inflammation of the OLETF rat retinas.

Key words: Interleukin 18, retina, diabetes mellitus, interferon- γ , OLETF rat

1 INTRODUCTION

The prevalence of diabetes mellitus, a common metabolic disorder, is increasing rapidly. Currently, the disease affects more than 190 million people worldwide, and this number will continue to rise in the near future^{1,2}. Among the factors responsible for the increasing prevalence of diabetes mellitus are obesity, the consumption of energy-dense diets, and low levels of physical activity³.

Diabetic retinopathy, one of the secondary complications of diabetes mellitus, is the leading causes of blindness in the industrialized world. There is emerging evidence to support the notion that inflammation in the retina, characterized by the activation of microglia and astroglia, is in-

involved in the pathogenesis of diabetic retinopathy. Therefore, diabetic retinopathy is a chronic, low-grade inflammatory disease⁴⁻⁶. Animal models, as well as clinical studies, have reported the idea that inflammation contributes to the development of diabetic retinopathy^{7,8}. Furthermore, increased leucostasis has been reported in the retinas of diabetic animals⁹, along with an increased expression of intercellular adhesion molecule 1 (ICAM-1)¹⁰ and platelet activation¹¹. Elevated levels of tumor necrosis factor alpha (TNF- α) in epiretinal membranes have been reported in diabetic patients¹², together with increased levels of the proinflammatory cytokine interleukin (IL) 1 β in retinal cell cultures exposed to high glucose concentra-

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tions^{13, 14}). In addition, various clinical and experimental studies have reported elevated concentrations of cytokines such as IL-8, IL-2, IL-1 α , IL-6, and interferon- γ (IFN- γ) in serum, vitreous, aqueous humor, and epiretinal membrane samples from subjects with diabetic retinopathy^{15–21}. On the other hand, IL-18 also plays an important role in inflammatory action^{22, 23}, and serum IL-18 concentrations are reported to be increased in individuals with human type 2 diabetes mellitus, in obese individuals, and in individuals with polycystic ovary syndrome^{24–26}. The infusion of glucose into normal volunteers and individuals with impaired glucose tolerance induces an acute increase in serum IL-18 concentrations²⁷. Furthermore, it has been reported that the expression of IL-18 mRNA in the retinas of streptozotocin-induced diabetic rats as an animal model for insulin-dependent diabetes mellitus is enhanced at 24 weeks after the onset of diabetes mellitus²⁸. However, to our knowledge, the effect of long-term (chronic) and acute hyperglycemia on IL-18 and IFN- γ production in the retina in the case of non-insulin-dependent diabetes mellitus has not yet been reported, and its elucidation is important.

IL-18 is a pleiotropic cytokine belonging to the IL-1 family^{22, 23}. It is expressed as an inactive 24-kDa proform that is cleaved by caspase-1 (IL-1 β converting enzyme, a cysteine protease) to an 18-kDa active form^{22, 23}. Released mature IL-18 exerts its effects upon binding to its cognate receptor (IL-18R)²⁹. Two subunits of IL-18R have been characterized as belonging to the IL-1R family. An IL-1R related protein (IL-1Rrp or IL-1 R5) was identified as a low-affinity receptor for IL-18 and renamed IL-18 R α ²⁹. The second subunit, IL-18R β does not bind IL-18 directly, but the β chain increases IL-18 binding affinity and is necessary for initiating signal transduction in target cells³⁰. The binding of mature IL-18 to IL-18R leads to the production of IFN- γ ³¹.

In the present study, we determined the expression of IL-18 in in diabetes mellitus retinas using Otsuka Long-Evans Tokushima Fatty (OLETF) rats, a model of type 2 diabetes mellitus. In addition, we investigated whether hyperglycemia relates to the expression of IL-18 and IFN- γ in the retinas of OLETF rats.

2 EXPERIMENTAL

2.1 Animals

Long-Evans Tokushima Otsuka (LETO) rat is widely used as a control rat of an OLETF rat (the food intake, water consumed, body weight, Glu, TG and Cho levels in LETO rats were similar to those in Wistar rats). Male LETO and OLETF rats at 10, 38 and 60 weeks of age were used in this study, and the LETO and OLETF rats were obtained from Otsuka Pharmaceutical Co., Ltd., and housed under standard conditions (12 h/d fluorescent light (07:00–19:00),

25°C room temperature) with free access to a commercial diet (CE-2, Clea Japan Inc., Tokyo, Japan) and water. All procedures were performed in accordance with the guidelines of the Kinki University Faculty of Pharmacy Committee for the Care and Use of Laboratory Animals and the Association for Research in Vision and Ophthalmology resolution on the use of animals in research.

2.2 Blood test for diabetes mellitus

Rats were fasted for 12 hours, after which blood was drawn from a tail vein at AM 9:00 without anesthesia, and plasma glucose (Glu), triglycerides (TG), total cholesterol (Cho), insulin and HbA1c levels were measured. The Glu and TG levels were determined using an Accutrend GCT (Roche Diagnostics, Mannheim, Germany). Cho levels were measured by the cholesterol oxidase method and the phosphotungstate-magnesium salt method using a Cholesterol E-Test Kit (Wako, Osaka, Japan). Insulin levels were measured using an ELISA Insulin Kit according to the manufacturer's instructions (Morinaga Institute of Biological Science Inc., Kanagawa, Japan). Briefly, monoclonal antibodies specific for rat insulin were pre-coated onto microplates, standards and samples were pipetted into the wells, and the microplates were incubated at 4°C for 2 h. After washing to remove unbound materials, rat insulin antibodies were added to the wells at room temperature for 30 min. After washing, the substrates were added. The enzyme reactions yielded blue products that turned yellow when the stop solutions were added. The absorbance was measured with a microplate reader (BIO-RAD, California, USA) at 450 nm. HbA1c levels were determined using an HLC-723GHbV (Tosoh Corp., Tokyo, Japan), which is a fully automated HPLC analyzer that uses a non-porous cation-exchange polymer that can separate the stable and labile Schiff fractions of HbA1c, and shows no interference by carbamylated hemoglobin. Blood samples were collected into EDTA-containing tubes from a tail vein. The eluted hemoglobin fractions are monitored at 415 nm as well as at 500 nm as a reference wavelength, and the analysis time was 2.2 min per sample. All analyses were performed according to the manufacturer's instructions.

2.3 RNA preparation

Total RNAs were extracted from the retinas of 60-week-old LETO and OLETF rats by the acid guanidium thiocyanate-phenol-chloroform extraction method³² using Trizol reagent (Life Technologies Inc., Rockville, USA). The purity and concentrations of RNA were determined spectrophotometrically. The OD₂₆₀/OD₂₈₀ values of all RNAs used were greater than 1.8, which indicates low protein contamination and high RNA purity.

2.4 Quantitative real-time reverse transcriptase-polymerase chain reaction (RT-PCR)

The RT reaction was performed using an RNA PCR Kit (AMV Ver 3.0, Takara Bio Inc., Shiga, Japan). One microgram of total RNA was mixed with 3 µl of 10 mM Tris-HCl buffer (pH 8.3) containing 5 mM MgCl₂ and 50 mM KCl. The following components were then added to give a final volume of 10 µl: 1 unit/µl RNase inhibitor, 10 mM deoxynucleotide triphosphate, 2.5 units/µl reverse transcriptase, and 0.125 µM oligo dT-adaptor primer. The RT reaction was performed at 42°C for 15 min, followed by 5 min at 95°C. The PCR reactions were performed using LightCycler FastStart DNA Master SYBR Green I according to the manufacturer's instructions (Roche Diagnostics Applied Science, Mannheim, Germany). Briefly, 2 µl of cDNA was mixed with 2 µl of reaction mixture, LightCycler FastStart DNA Master SYBR Green I Reaction Mix, containing FastStart Taq DNA Polymerase, reaction buffer, MgCl₂, SYBR Green I dye, and deoxynucleotide triphosphate mix. The following components were then added to give a final volume of 20 µl: 10 pmol specific primers for IL-18, IL-18Rα, IL-18Rβ, IL-18 binding protein (IL-18BP), caspase-1 or glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The primers used are summarized in Table 1. The PCR conditions were 95°C for 10 min, 50 cycles of 95°C for 10 s (denaturing), 53 (IL-18, IL-18BP), 55 (IL-18Rβ), 57 (IL-18Rα, caspase-1) or 60 (GAPDH)°C for 10 s (annealing), and 72°C for 5 s (extension). The quantities of PCR products were measured fluorometrically in a real-time manner using a LightCycler DX 400 (Roche Diagnostics Applied Science, Mannheim, Germany). After the completion of the PCR reactions, dissociation curves of the PCR products were generated using the LightCycler Software Version 4.0 program to detect nonspecific amplifica-

tion, including primer-dimers, and to ascertain the quality of the amplification data. The differences in the threshold cycles for GAPDH and other groups (IL-18, IL-18Rα, IL-18Rβ, IL-18BP and caspase-1) were used to calculate the levels of mRNA expression in LETO and OLETF rats³³.

2.5 Western blot analysis

The retinas of 60-week-old LETO and OLETF rats were homogenized in saline on ice, and centrifuged at 1,500 rpm for 10 min at 4°C. The resultant supernatants were suspended in a buffer comprising 1.25 mmol/l Tris-HCl, pH 6.8, 0.4% sodium dodecyl sulfate (SDS), 0.2% glycerol, 0.1% 2-mercaptoethanol and 0.004% bromophenol blue, and boiled for 3 min. A sample of total protein (10 µg) was separated in a 15% polyacrylamide SDS gel, and the proteins were then transferred to polyvinylidene difluoride membranes (BIO-RAD, California, USA) using a semi-dry transfer cell (Trans-Blot SD Semi-Dry Electrophoretic Transfer Cell, BIO-RAD, California, USA). The transfer buffer used in the system contained 25 mmol/l Tris-HCl, 191 mmol/l glycine, 20% methanol, and 0.0375% SDS. After transfer, nonspecific sites on the membranes were blocked with 5% non-fat dry milk in Tris-buffer (20 mmol/l Tris-HCl, and 500 mmol/l NaCl, pH 7.5). The blots were probed with 0.31 mg/l goat anti-rat IL-18 polyclonal antibody (Promega, Wisconsin, USA) or with 0.17 mg/l rabbit anti-rat GAPDH polyclonal antibody (IMGEX, California, USA) for 2 h at room temperature. After washing with Tris-buffer containing 0.05% Tween 20, the membranes were incubated with secondary alkaline-phosphatase conjugated anti-goat IgG (1:7000 dilution, Promega, Wisconsin, USA) or alkaline-phosphatase conjugated anti-rabbit IgG (1:7000 dilution, Promega, Wisconsin, USA) for 2 h at room temperature, washed with Tris-buffer containing 0.1% Tween 20, and in-

Table 1 Sequences of Primers used for Quantitative RT-PCR Analysis.

Primer		Sequence (5' -3')	GenBank Accession No.
IL-18	FOR	CGCAGTAATACGGAGCATAAATGAC	NM_019165
	REV	GGTAGACATCCTTCCATCCTTCAC	
IL-18Rα	FOR	AGCAGAAAGAGACGAGACACTAAC	XM_237088
	REV	CTCCACCAGGCACCACATC	
IL-18Rβ	FOR	GACCACAGGATTTAACCATTTCAGC	AJ550893
	REV	AGCAGGACCTAGTGTGATGATG	
IL-18BP	FOR	TTGGTGGGTCCTGCTTCTATATG	AF154569
	REV	GGTCAGCGTTCCATTCAGTG	
Caspase-1	FOR	TGAAGATGATGGCATTAAAGAAGGC	NM_012762
	REV	CAAGTCACAAGACCAGGCATATTC	
GAPDH	FOR	ACGGCACAGTCAAGGCTGAGA	NM_017008
	REV	CGCTCCTGGAAGATGGTGAT	

cubated with a stabilized substrate for alkaline phosphatase (Promega, Wisconsin, USA)³³⁾.

2.6 Protein measurement

Protein levels in the retinas of LETO and OLETF rats at 10 and 60 weeks of age were determined according to the method of Bradford³⁴⁾ using a Bio-Rad Protein Assay Kit (BIO-RAD, California, USA) with bovine serum albumin as the standard.

2.7 Assay of IL-18 levels in LETO and OLETF rat retinas

Retinas from LETO and OLETF rats at 10 and 60 weeks of age were homogenized in ultrapure water. The retinal homogenates were centrifuged at 1500 rpm for 10 min at 4°C, and the supernatants were used for the measurement of IL-18 levels using an Endogen[®] Rat IL-18 ELISA Kit according to the manufacturer's instructions (Invitrogen Cor., Camarillo, CA). Briefly, monoclonal antibodies specific for rat IL-18 were pre-coated onto microplates. Standards and samples were pipetted into the wells, and the microplates were incubated at room temperature for 2 h. After washing to remove unbound materials, biotinylated antibodies were added to the wells. After washing, streptavidin-HRP solutions were added, and the wells were washed to remove unbound materials after which the substrates were added. The enzyme reactions yielded blue products that turned yellow when the stop solutions were added. The absorbance was measured with a microplate reader (BIO-RAD, California, USA) at 450 nm³³⁾. The detection area of IL-18 was 15.6-1000 pg/ml, and the IL-18 levels in this study were within detection area.

2.8 Assay of IFN-γ levels in LETO and OLETF rat retinas

Retinas from LETO and OLETF rats at 10 and 60 weeks of age were homogenized in ultrapure water. The retinal homogenates were centrifuged at 1500 rpm for 10 min at 4°C, and the supernatants were used for the measurement of IFN-γ levels using an Endogen[®] Rat IFNγ ELISA Kit according to the manufacturer's instructions (Pierce Biotechnology Inc., Wisconsin, USA). Briefly, monoclonal antibodies specific for rat IFN-γ were pre-coated onto microplates. Standards and samples were pipetted into the wells, and the microplates were incubated at room temperature for 1 h. After washing to remove unbound materials, biotinylated antibodies were added to the wells. After washing, streptavidin-HRP solutions were added, and the wells were washed to remove unbound materials after which the substrates were added. The enzyme reactions yielded blue products that turned yellow when the stop solutions were added. The absorbance was measured with a microplate reader (BIO-RAD, California, USA) at 450 nm³³⁾. The detection area of IFN-γ was 8-500 pg/ml, and The IFN-γ levels in this study were within detection area.

2.9 Statistical analysis

All values are represented as mean ± standard error of the mean (S.E.). Unpaired Student's or Aspin-Welch's *t*-tests were used for statistical analysis, and multiple groups were evaluated by one-way analysis of variance (ANOVA) followed by Dunnett's multiple comparison. *P* values less than 0.05 were considered significant. The number of experiments performed is given in the Table and Figure legends.

3 RESULTS

3.1 Changes in food intake, water consumption, body weight and blood markers for diabetes mellitus in LETO and OLETF rats

Table 2 shows the changes in food intake, water consumption, body weight and blood markers for diabetes of LETO and OLETF rats at 10, 38 and 60 weeks of age. The food and water intakes of OLETF rats were higher than those of LETO rats. The body weights of the 38-week-old OLETF rats were 1.27-fold those of LETO rats at the same age. In addition, the Glu, TG, Cho and insulin levels of the OLETF rats were significantly higher than those of the LETO rats. In 60-week-old OLETF rats, the Glu, TG and Cho levels were also significantly higher than those of the same age LETO rats. On the other hand, the body weight and plasma insulin levels were lower, and HbA1c levels were significantly higher than in 60-week-old LETO rats.

3.2 Expression of IL-18 mRNA and protein in the retinas of LETO and OLETF rats

Figure 1 shows the expression levels of the mRNAs for IL-18, IL-18Rα, IL-18Rβ, IL-18BP and caspase-1 in the retinas of LETO and OLETF rats aged 60 weeks as determined by the quantitative real-time PCR method. In the retinas of OLETF rats, high expression levels of the mRNAs for IL-18, IL-18Rα, IL-18Rβ, IL-18BP and caspase-1 were observed in comparison with those in LETO rats. Furthermore, the mRNA expression levels of IL-18 and caspase-1 in the retinas of OLETF rats were significantly higher than those in LETO rats. Figure 2 shows the expression of mature IL-18 in 60-week-old OLETF rat retinas as determined by western blot. Although no expression of mature IL-18 (18-kDa) was observed in the retinas of 60-week-old LETO rats, OLETF rats of the same age showed clear expression of mature IL-18. On the other hand, in the rat which was perfused by cold saline, the significantly different of IL-18 mRNA was not observed between LETO and OLETF rats aged 60 weeks (LETO; 8.0 ± 1.1 , OLETF; 9.7 ± 1.5 , $\times 10^{-5}$, means ± S.E. of 3 experiments), and the expression of mature IL-18 protein was not detected by western blot.

Table 2 Body Weight and Some Blood Test Values for Diabetes Mellitus in 10-, 38- and 60-Week-Old LETO and OLETF Rats.

	10-week-old		38-week-old		60-week-old	
	LETO	OLETF	LETO	OLETF	LETO	OLETF
Food intake (g/day/rat)	22.3 ± 3.1	26.1 ± 1.3*	26.9 ± 2.2	38.5 ± 1.6*	29.7 ± 1.9	38.9 ± 3.2*
Water consumed (ml/day/rat)	30.2 ± 1.2	28.8 ± 0.9	38.6 ± 2.8	82.3 ± 2.9*	39.4 ± 2.6	79.8 ± 3.6*
Body weight (g)	220.6 ± 8.1	278.3 ± 6.7*	488.6 ± 14.2	621.3 ± 19.7*	466.9 ± 13.9	408.3 ± 14.9*
Glu (mg/dl)	103.8 ± 2.9	124.3 ± 5.3*	119.3 ± 4.9	213.5 ± 15.7*	127.3 ± 3.2	238.1 ± 18.3*
TG (mg/dl)	101.6 ± 7.6	114.5 ± 10.1	128.0 ± 9.3	419.8 ± 22.2*	119.8 ± 7.6	335.0 ± 8.1*
Cho (mg/dl)	84.7 ± 21.7	106.3 ± 9.6	101.4 ± 11.4	209.2 ± 11.1*	93.8 ± 21.2	253.3 ± 20.3*
Insulin (ng/dl)	95.3 ± 9.5	99.5 ± 3.0	105.5 ± 11.6	237.4 ± 26.6*	103.7 ± 7.9	68.7 ± 11.0*
HbA1c (%)	—	—	—	—	2.9 ± 0.07	4.5 ± 0.23*

The Glu and TG levels were determined using an Accutrend GCT; Cho levels were measured using a Cholesterol E-Test Kit. Insulin was determined using the ELISA method, and HbA1c was analyzed by HLC-723GHbV. The data are presented as means ± S.E. of 8 independent rats. *¹ $p < 0.05$ vs. 10-week-old LETO rats for each category. *² $p < 0.05$ vs. 38-week-old LETO rats for each category. *³ $p < 0.05$ vs. 60-week-old LETO rats for each category.

Fig. 1 Expression of IL-18-Related Genes in the Retinas of 60-week-old LETO and OLETF Rats. mRNA expression levels were determined by the quantitative real-time RT-PCR method. A, IL-18; B, IL-18R α ; C, IL-18R β ; D, IL-18BP; E, caspase-1. Open columns, LETO rats; closed columns, OLETF rats. The data are presented as means ± S.E. of 4 independent rats. * $p < 0.05$ vs. LETO rats. **Fig. 2** Expression of the Mature IL-18 Protein in the Retinas of 60-week-old LETO and OLETF Rats. The mature IL-18 protein was detected by Western blot analysis.

3.3 Levels of IL-18 and IFN- γ production in the retinas of LETO and OLETF rats

Figure 3 shows the levels of IL-18 and IFN- γ in the retinas of LETO and OLETF rats aged 10 and 60 weeks as determined by ELISA. The levels of IL-18 and IFN- γ in the retinas of 10-week-old rats did not differ between LETO and OLETF rats, and both IL-18 and IFN- γ levels in the retinas of 60-week-old OLETF rats were significantly greater than in the retinas of 60-week-old LETO rats. The IFN- γ levels increased with increasing IL-18 levels in the retinas of 60-week-old OLETF rats (Fig. 4). Table 3 shows the correlation coefficients (r) between the IL-18 levels and blood test values for diabetes mellitus (Glu, TG, Cho, insulin and HbA1c) in 60-week-old OLETF rats. Body weight, Cho, TG, Cho and insulin levels showed no significantly relationship to IL-18 and IFN- γ levels. Although, the IL-18 and IFN- γ levels in retinas of 60-week-old OLETF rats increased with an increase in Glu level, the change was not statistically significant. Both of the IL-18 and IFN- γ levels of 60-week-old OLETF rats increased with the in-

Fig. 3 IL-18 (A) and IFN- γ (B) Levels in the Retinas of 10- and 60-week-old LETO and OLETF Rats. IL-18 and IFN- γ levels were determined using the ELISA method. Open columns, LETO rat retinas; closed columns, OLETF rat retinas. The data are presented as means $\pm$ S.E. of 8 independent rat retinas. * $p < 0.05$, vs. LETO rats.

Fig. 4 Relationship between the Levels of IL-18 and IFN- γ in the Retinas of 60-week-old OLETF Rats. * $p < 0.05$.

Table 3 Correlation Coefficients (r) among IL-18 and IFN- γ Levels and Body Weight, Glu, TG, Cho, Insulin and HbA1c Levels in 60-Week-Old OLETF Rats.

Relationship	IL-18	IFN- γ
vs. body weight	0.10	0.24
vs. Glu	0.67	0.49
vs. TG	0.35	0.50
vs. Cho	0.02	-0.18
vs. insulin	0.40	0.54
vs. HbA1c	0.75*	0.77*

The Glu and TG levels were determined using an Accutrend GCT; Cho levels were measured by Cholesterol E-Test Kit. Insulin, IL-18 and IFN- γ levels were determined using the ELISA method, and HbA1c was analyzed by HLC-723GHbV. The data represent mean values for 8 independent rats. * $p < 0.05$.

crease in HbA1c, and this relationship was significant (Table 3). Table 4 shows the effect of acute hyperglycemia on IL-18 and IFN- γ production in 60-week-old OLETF rats 60 min after the oral administration of a glucose solution (3.0 g/kg). The rapid rise in Glu following the oral administration of glucose did not affect the IL-18 and IFN- γ levels in the retinas of LETO rats. Whereas, the IL-18 and IFN- γ levels in the retinas of OLETF rats rose significantly following the oral administration of glucose (Table 4). On the other hand, at 60 min after the oral administration of glucose, the enhancement of IL-18 mRNA level was observed in the retina of 60-week-old OLETF rats perfused by cold saline (without glucose; 9.4 ± 1.8 , with glucose; 13.5 ± 1.7 , $\times 10^{-5}$, means $\pm$ S.E. of 3 experiments), and IL-18 levels in the retina of 60-week-old OLETF rats with oral administration of glucose was also higher than that without oral administration of glucose (without glucose; 0.9 ± 0.2 , with glucose; 1.5 ± 0.3 , pg/mg protein, means $\pm$ S.E. of 3 experiments), however, the expression of IL-18 mRNA and IL-18 levels were a little in comparison with blood in the retina of 60-week-old OLETF rats.

4 DISCUSSION

IL-18, which induces IFN- γ production, is a well-known pleiotropic cytokine that regulates innate and acquired immune responses, and plays an important role in inflammatory action^{22, 23}. In this study, we determined the expression of IL-18 in diabetic retinas using OLETF rats, a model of type 2 diabetes mellitus. In addition, we investigated whether hyperglycemia relates to the expression of

Table 4 Changes in IL-18 and IFN- γ Levels in 60-Week-Old LETO and OLETF Rats in Response to an Oral Glucose Tolerance Test.

	LETO		OLETF	
	Purified water	Glucose	Purified water	Glucose
Glu (mg/dl)	125.3 $\pm$ 7.9	228.9 $\pm$ 16.7 ^{*1}	225.5 $\pm$ 30.1	482.6 $\pm$ 33.1 ^{*1, 2}
IL-18 (pg/mg protein)	3.5 $\pm$ 0.8	3.6 $\pm$ 0.9	6.3 $\pm$ 1.2	9.9 $\pm$ 1.6 ^{*1, 2}
IFN- γ (pg/mg protein)	5.8 $\pm$ 0.7	5.8 $\pm$ 1.0	9.6 $\pm$ 1.1	15.1 $\pm$ 2.7 ^{*1, 2}

The Glu, IL-18 and IFN- γ levels in 60-week-LETO and OLETF rats were measured 60 min after the oral administration of glucose (3.0 g/kg). Glu was determined using an Accutrend; IL-18 and IFN- γ levels were measured by the ELISA method. The data are presented as means $\pm$ S.E. of 3 independent rat retinas.

^{*1} $p < 0.05$ vs. purified water-administered LETO rats for each category. ^{*2} $p < 0.05$ vs. purified water-administered OLETF rats for each category.

IL-18 and IFN- γ in OLETF rat retinas.

In studies to identify changes in retinal levels of IL-18 in diabetes mellitus, the selection of the experimental animal is very important. Animal models used to investigate the ophthalmological secondary complications of diabetic mellitus include streptozotocin-induced diabetic rats as an animal model for insulin-dependent diabetes mellitus, and galactose fed rats as an animal model for sugar cataracts³⁵⁻³⁹. Hyperglycemia is present in streptozotocin-induced diabetic rats, but not in galactose fed rats. However, the general pathophysiology of these models differs from that of non-insulin-dependent diabetes mellitus in humans. On the other hand, the general pathophysiology of GK (Goto-Kakizaki) rats, which are used as a model animal for type 2 diabetes mellitus, is similar to that of humans⁴⁰. However, GK rats develop type 2 diabetes mellitus without metabolic syndrome. The OLETF rat is known as a model animal for type 2 diabetes mellitus *via* a metabolic syndrome⁴¹. Nearly 100% of male OLETF rats develop a diabetic syndrome by 25 weeks of age. The food intake and water consumption in OLETF rats are higher, and the body weight and TG levels of OLETF rats are significantly increased than that in LETO rat. This enhancement of TG levels causes insulin resistance. Thereby, hyperglycemia and hyperinsulinemia are exhibited in the early phases of the disease, which makes them a suitable model for human type 2 diabetes mellitus⁴²⁻⁴⁵. With continued aging, the rats eventually develop hypoinsulinemia as a result of islet beta cell deterioration^{42, 45}, and plasma insulin levels in OLETF rats over 60 weeks of age are lower than in LETO rats of the corresponding age used as normal controls^{46, 47}. In addition, our previous studies have shown that a delay in corneal wound healing, lens opacification *via* IL-18 and IFN- γ expression and retinal damage can be observed in 60-week-old OLETF rats⁴⁸⁻⁵⁰, and Miyamura *et al.*⁵⁰ have demonstrated the usefulness of OLETF rats as a model of diabetic retinal disease. The basement membranes of the retinal capillaries of 56-week-old OLETF rats are significantly thicker than in LETO rats, and the ratio of the peri-

cyte area to the capillary cross-section area is significantly lower. The endothelial cell cytoplasm degenerates, and the vascular corrosion cast of a 56-week-old OLETF rat shows caliber irregularity, narrowing, tortuosity and loop formations of the capillaries. Furthermore, the electroretinograms (ERG) are similar to those seen in diabetic patients^{51, 52}. From these reports, OLETF rats may provide a better model than streptozotocin-induced diabetic rats or GK rats for studies to clarify the significance of IL-18 in the retina of diabetes mellitus.

The body weights of 38-week-old OLETF rats were 1.27-fold higher than those of 38-week-old LETO rats. Plasma Glu, TG and Cho levels in OLETF rats increased with age, and the plasma insulin levels in 38-week-old OLETF rats were also higher than those in 38-week-old LETO rats. These results indicate that the 38-week-old OLETF rats had developed diabetes mellitus *via* insulin resistance. Consistent with the results in 38-week-old OLETF rats, the body weights and plasma insulin levels of 60-week-old OLETF rats were lower than those of 60-week-old LETO rats (Table 2). Hirashima *et al.*⁴⁶ reported atrophy or the disappearance of beta cells in OLETF rats older than 60 weeks of age, and found that plasma insulin levels were lower than in LETO rats⁴⁷. These findings suggest that the type 2 diabetes mellitus in 60-week-old OLETF rats has reached a fairly advanced stage, and that the changes may be due to the deterioration of islet beta cells with the progression of the disease. It is known that early symptoms of diabetic retinopathy are seen in 60-week-old OLETF rat^{51, 52}, and the development of secondary ophthalmological complications of diabetes mellitus such as in the cornea and lens show that the retina is also effected by hyperglycemia^{49, 51-53}. Therefore, we confirmed the onset of cataracts, and used 60-week-old OLETF rats in which lens opacification can be observed in this study (some data are showed in Ref.⁴⁹). These results support the use of 60-week-old OLETF rats as a model for the early phases in the development of diabetic retinopathy.

The IL-18 levels in the retinas of 10-week-old OLETF

rats, which do not show signs of secondary ophthalmological complications of diabetes mellitus, were approximately 3.3 pg/mg protein, similar to the level in LETO rats (Fig. 3). On the other hand, higher expression levels of the mRNAs for IL-18, IL-18R α , IL-18 β and caspase-1 were observed in the retinas of 60-week-old OLETF rats that did display secondary ophthalmological complications of diabetes mellitus than in LETO rats, and the elevations were significant in the case of the IL-18 and caspase-1 mRNAs (Fig. 1). The expression of the mature IL-18 protein in the retinas of 60-week-old OLETF rats was also observed to be significantly higher than in LETO rats (Fig. 2 and 3A). The IFN- γ level in the retinas of OLETF rats was also higher than in LETO rats, and a close relationship was observed between the levels of IL-18 and IFN- γ (Fig. 3B and 4). In addition, the 60-week-old OLETF rats by dietary restriction did not develop the diabetic mellitus (Glu; 129.0 ± 6.8 mg/dl, insulin; 101.5 ± 8.1 ng/dl, means $\pm$ S.E. of 3 experiments), and the IL-18 and IFN- γ levels in retina of 60-week-old OLETF rats without diabetic mellitus by dietary restriction (IL-18; 3.5 ± 1.3 , IFN- γ ; 5.7 ± 1.4 , means $\pm$ S.E. of 3 experiments) was similar to LETO rats at 60 weeks of age. Retinal inflammation is associated with T lymphocyte infiltration⁵⁴⁾, and an increase in IFN- γ is a classical part of the Th1 cytokine response. Geiger *et al.* reported that transgenic mice expressing IFN- γ in the retina developed eye inflammation and photoreceptor loss⁵⁵⁾. Furthermore, IFN- γ has not been detected in the vitreous or aqueous fluid of patients with proliferative diabetic retinopathy¹⁵⁾. Therefore, it seems likely that the retina has a selective response to the diabetic insult. These results suggest that IFN- γ production *via* IL-18 may cause inflammation in the retinas of 60-week-old OLETF rats. On the other hand, the expression of the mRNA for IL-18BP, a protein that inhibits the activation of IL-18, in the retinas of OLETF rats also tended to be higher than in LETO rats (Fig. 1). Soluble IL-18BP, which has recently been purified and cloned from serum and urine, shows no homology to IL-18R, but is a specific, functional inhibitor of IL-18 that may act as an endogenous inhibitor of circulating IL-18^{56, 57)}. Hurgin *et al.*⁵⁸⁾ reported that an elevation in IL-18BP expression is induced by IFN- γ . Therefore, the elevation in the expression of IL-18BP mRNA in the retinas of OLETF rats may be caused by the production of IFN- γ through the activation of IL-18.

It is very important to elucidate the precise process by which IL-18 enhancement occurs in the retinas of OLETF rats. At present, we have demonstrated changes in IL-18 and IFN- γ levels in 60-week-LETO and OLETF rats 60 min after the oral administration of glucose (3.0 g/kg, Table 4). The Glu levels in LETO rats administered the glucose solution were similar to those in OLETF rats administered purified water. However, the IL-18 and IFN- γ levels in LETO rats did not increase following the administration of glucose solution, and the IL-18 and IFN- γ levels in LETO

rats administered glucose solution were lower than those in OLETF rats administered purified water. Consistent with the results in LETO rats, the IL-18 and IFN- γ levels in the retina of OLETF rats were increased by the oral administration of glucose (Table 4), and the relationships (r) between the Glu and IL-18 or IFN- γ levels in the retina of OLETF rats administered glucose solution (vs. IL-18, 0.77; IFN- γ vs., 0.78) were higher than in OLETF rats administered purified water (vs. IL-18, 0.65; IFN- γ vs., 0.59). These results suggest that chronic hyperglycemia may enhance the immune activation, as reflected in the increased IL-18 and IFN- γ levels, in diabetes mellitus. In addition, we measured HbA1c levels, and demonstrated relationships between it and the IL-18 and IFN- γ levels. It is known that retinal HbA1c levels in rats are lower than in humans with type 2 diabetes mellitus, and Taniguchi *et al.*⁵⁹⁾ have shown that HbA1c levels in 25-week-old LETO and OLETF rats are approximately 2.9% and 3.5%, respectively. In this study, the HbA1c levels in 60-week LETO and OLETF rats were similar to those reported by Taniguchi *et al.*,⁵⁹⁾ and were higher in OLETF rats than in LETO rats. The relationships between the levels of IL-18 or IFN- γ and HbA1c are close, and are closer than the relationships between the levels of IL-18 or IFN- γ and Glu (Table 3). Therefore, it is hypothesized that long-term hyperglycemia induces an enhancement in the expression of genes that lead to IL-18 production (IL-18, IL-18R and caspase-1), so that IFN- γ levels *via* IL-18 increase in retinas.

The IL-18 levels in serum of 60-week-old OLETF rats were also significantly higher than 10-week-old OLETF rats (10 weeks of age; 3.3 ± 1.3 , 60 weeks of age; 8.9 ± 1.7 , pg/mg protein, means $\pm$ S.E. of 3 experiments). Whereas, the different of IL-18 mRNA was not observed between LETO and OLETF rats aged 60 weeks perfused by cold saline, and the expression of mature IL-18 protein was not detected in the retina of 60-week-old OLETF rat perfused by cold saline. These results show that the inflammatory cells in the blood relate the enhancement of IL-18 in the retina. On the other hand, the IL-18 levels in the retinas of 60-week-old OLETF rats rose significantly following the oral administration of 3.0 g/kg glucose (Table 4); however, the rapid rise in Glu at 60 min after the oral administration of glucose did not affect the IL-18 levels in the serum of 60-week old OLETF rats (without glucose; 8.6 ± 1.3 , with glucose; 8.9 ± 1.6 , pg/mg protein, means $\pm$ S.E. of 3 experiments). Moreover, the IFN- γ is not detected by used kit in the serum of 10- and 60-week-old OLETF rats. In addition, at 60 min after the oral administration of glucose, the expression of IL-18 mRNA and IL-18 levels in the retina without blood were a little than blood in the retina of 60-week-old OLETF rats. These results suggest that the cells in the blood locally caused the IL-18 and IFN- γ in the retina under the rapid enhancement of Glu. It was known that the retina of OLETF rats was injured, and the inflammatory cells gather

in the injured area^{51, 52}. Taken together, it was possible that the inflammatory cells were gather in the blood of retina, and rapidly enhanced Glu may accelerate the release of IL-18 from the inflammatory cells in retinal blood vessel, resulting in enhancement of IL-18 in retina of the OLETF rats in this study.

Further studies are needed to elucidate the release and distribution of IL-18 in the retina of OLETF rats using histochemical procedure. In addition, it is important to clarify whether treatment with antihyperglycemic drugs can prevent retinopathy in diabetic patients. Therefore, we next plan to investigate the distribution of IL-18 in the retina of OLETF rats using histochemical procedure, and demonstrate the effects of orally administered antihyperglycemic drugs on IL-18 and IFN- γ production in the retinas of OLETF rats with diabetes mellitus.

5 CONCLUSION

The present study, we demonstrate the enhancement of IL-18 expression in the retinas of OLETF rats, a spontaneous diabetic animal model with metabolic syndrome. The expression levels of IL-18 mRNA and protein in the retinas of 60-week-old OLETF rats are higher than in LETO rats of the corresponding age, and the inflammatory cells in the blood relate the enhancement of IL-18 in the retina. In addition, we report the relationship between hyperglycemia and IL-18 production in the retinas of OLETF rats. A close relationship was observed among the levels of IL-18, IFN- γ and HbA1c, and chronic hyperglycemia may accelerate the release of IL-18 and IFN- γ from inflammatory cells in retinal blood vessel. It is possible that IFN- γ production *via* IL-18 in the retinas of 60-week-old OLETF rats is caused by hyperglycemia, and this may play a role in the inflammation observed in OLETF rat retinas.

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