

The Development of Diabetic Retinopathy in Goto-Kakizaki Rat and the Expression of Angiogenesis-Related Signals

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Abstract

The Goto-Kakizaki (GK) rat is a genetic model of type 2 diabetes. Diabetic retinopathy (DR) is a common complication of diabetes. In this study, we observed the development of DR in GK rats and the expression of some angiogenesis-related signals. GK rats were housed for 5, 6 and 7 months. Results of retinal vessels stained by cluster of differentiation 31 (CD31) showed that the number of retinal vessels was increased in GK rats at both 6 and 7 months. Retinal histological observation also evidenced such increase. Retinal mRNA expressions of hypoxia inducible factor-1 α (HIF-1 α), vascular endothelial growth factor (VEGF), VEGFB and its receptors (VEGFR1/2), basic fibroblast growth factor (bFGF), and platelet-derived growth factor (PDGF) A/B were increased in GK rats at both 6 and 7 months. Retinal mRNA expressions of matrix metalloproteinase (MMP) 2/9 and insulin-like growth factor 1 (IGF-1) were increased at 7 months. Retinal mRNA expression of pigment epithelium-derived factor (PEDF) was increased in GK rats at 6 months. Serum contents of VEGF, bFGF, PDGFA, MMP2/9, IGF-1, PEDF were increased in GK rats at both 6 and 7 months, while PDGFB was increased at 7 months. In summary, our results indicate that retinal angiogenesis occurred in GK rats at 6 and 7 months, and the expressions of some angiogenesis related factors were increased during the development of DR in GK rats.

Key Words: angiogenesis, diabetic retinopathy, Goto-Kakizaki rat, MMP2/9, PDGFA/B, VEGF

Introduction

Diabetic retinopathy (DR) is one of the most common and serious complications of diabetes mellitus, and also is the most common reason of visual loss in diabetics (41). DR is badly affecting the life quality of diabetics. Report demonstrated that nearly all people with type 1 and more than half with type 2 diabetes will develop retinopathy (14). In recent years, the incidence of DR is increased, and DR has become one of the major causes of blindness in human beings.

The cause of DR is due to the microvascular changes in retina, and it is generally divided into two stages: non-proliferative (NPDR) and proliferative stages (PDR) (40). During the stage of NPDR, following key events were occurred: loss of retinal capillary pericytes, basement membrane thickening, and breakdown of the blood retinal barrier, which will lead to the ischemia of retina (11). With the disease progressed to PDR stage: retinal vessels were proliferated due to the lack of oxygen, and then these new vessels will bleed, and destroy the retina (11). Due

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to its complicated pathogenesis and its serious effect on life quality of diabetics, the study on the process of the development of DR has been a hotspot. Up to now, a variety of studies have found the critical roles of some signals in the development of DR, including pro-angiogenic growth factors such as vascular endothelial growth factor (VEGF), basic fibroblast growth factor (bFGF), platelet-derived growth factor (PDGF), *etc.*, pro-inflammatory cytokine tumor necrosis factor (TNF)- α , and some other cellular molecules (8, 12, 13, 18, 32, 33, 36).

The Goto-Kakizaki (GK) rat, generated from glucose-intolerant Wistar rats, is an experimental model for spontaneous type 2 diabetes (2, 24). There is already report demonstrated that the retinal mean circulation time was significantly prolonged, and retinal segmental blood flow was obviously reduced in GK rats of 1, 3, 5 month (27). Another report showed that in GK rats of 5 month, there were sparse microglia/macrophages detected in the sub-retinal space and numerous pores in the retinal pigment epithelial (RPE) cells, while all those phenomena become stronger in GK rat at 12 month, and protein kinase C ξ (PKC ξ) was involved in such process (30). Meanwhile, the altered endothelial/pericyte ratio was observed in retinas of GK rat at 8 month (3). All these studies demonstrate that GK rat appears to be a suitable model for experimental studies of DR. However, there are still various problems needed to be solved such as how about retinal angiogenesis and the involved signals during the development of PDR in GK rat. Thus, the present study was designed to observe the development of PDR in GK rats and the expression of some angiogenesis related signals.

Materials and Methods

Reagents

Cluster of differentiation 31 (CD31) antibody and fluorescein isothiocyanate (FITC) conjugated Anti-Rat IgG were purchased from BD Biosciences (Franklin Lakes, NJ, USA). PrimeScript[®] RT Master Mix and SYBR[®] Premix Ex Taq[™] were purchased from Takara (Shiga, Japan). Enzyme-linked immunosorbent assay (ELISA) kits were purchased from RapidBio (West Hills, CA, USA). Other reagents unless indicated were purchased from Sigma Chemical Co. (St. Louis, MO, USA).

Animal

The GK rats and normal Wistar rats weighting 190-230 g (8 weeks old) were purchased from the shanghai laboratory animal center of Chinese Academy of Science (Shanghai, China). The rats were housed

under controlled temperature of $23 \pm 2^\circ\text{C}$ and 50% of humidity. The light was lighting for 12 h one day. The rats were given a standard diet and enough water. All experiments were strictly followed the institutional animal care guidelines approved by Experimental Animal Ethical Committee of Shanghai University of Traditional Chinese Medicine.

Animal Treatments

GK rats were divided into three groups randomly: 5 months group, 6 months group, and 7 months group. Accordingly, normal Wistar rats were also divided into three groups randomly: 5 months group, 6 months group, and 7 months group. At 5, 6, 7 months old, the rats were anesthetized by sodium pentobarbital (40 mg/kg, *i.p.*), the blood sample was taken from the abdominal aorta, and the eyes were removed immediately.

Retinal Immunofluorescence Staining

Retinas were removed from eyes immediately and then incubated in 4% paraformaldehyde solution over night in 4°C . The next day moved the retinas to blocking buffer (5% BSA and 0.5% triton X-100 in PBS) and blocked for 3 h at room temperature, after that incubated the retinas with CD31 antibody for 1-2 days in 4°C . Washed retinas with washing buffer (0.5% triton X-100 in PBS), and further incubated with FITC-conjugated Anti-Rat IgG antibody for 2 h. After washing, removed retinas onto a slide glass, used gelatin for mounting and covered with a cover slip, and taken pictures under a fluorescence microscopy. The quantitative of the vessels was counted as described: two lines, which constitute a cross, were drawn in the central on the pictures. Then, the number of vessels cross these two lines was counted (16, 22).

Histological Assessment

The retinas were isolated from the normal Wistar rats and diabetic GK rats, and then fixed in 4% paraformaldehyde solution. Retinas were subsequently sectioned ($5 \mu\text{M}$), stained with haematoxylin and eosin, and examined under the microscopy (Olympus, Japan).

Real-Time Polymerase Chain Reaction (PCR) Analysis

The total RNA was extracted from rat retinas by using TRIZOL (Life Technologies, Carlsbad, CA, USA) reagent according to the manufacturer's protocol. The single strand cDNA was synthesized according to the manufacturer's instruction described

Table 1. The list of primers used in Real-time PCR analysis

Target	Gene ID	Primer	Sequence	Annealing Temperature (°C)
GAPDH	24383	FP	5'- TCCACCACCCTGTGCTGTA -3'	60
		RP	5'- ACCACAGTCCATGCCATCAC -3'	
VEGF	83785	FP	5'- CGCAGCTACTGCCATCCAAT -3'	60
		RP	5'- CCCACAGGGATTTTCTTGTCTT -3'	
VEGFB	89811	FP	5'- CCCTGTGTCCCAGTTTGATGG -3'	60
		RP	5'- CGAGTTGTTTGACCACATTACCCAT -3'	
VEGFR1	54251	FP	5'- CCTGATGGGCAAAGAATAACAT -3'	60
		RP	5'- ATTTGGACATCTAGGATTGTATTGG -3'	
VEGFR2	25589	FP	5'- TAGCACGACAGAGACTGTGAGG -3'	60
		RP	5'- TGAGGTGAGAGAGATGGGTAGG -3'	
HIF-1 α	29560	FP	5'- TAGACTTGAAATGCTGGCTCCCT -3'	60
		RP	5'- TGGCAGTGACAGTGATGGTAGGTT -5'	
MMP2	81686	FP	5'- AAAGATTGATGCCGTGTACGAGG -3'	60
		RP	5'- GGTAACCCAGGCTGGTCAGT -3'	
MMP9	81687	FP	5'- GCAAACCCTGCGTATTTCCATT -3'	60
		RP	5'- GATAACCATCCGAGCGACCTTT -3'	
bFGF	54250	FP	5'- ACCTGGCTATGAAGGAAGATGGAC -3'	60
		RP	5'- GAGTTTATACTGCCAGTTCGTTTC -3'	
IGF-1	24482	FP	5'- GCATTCGGAGGGCACCACAGAC -3'	60
		RP	5'- GTAGGCTTCAGCGGAGCACAGT -3'	
PDGFA	25266	FP	5'- CTGTAACACCAGCAGCGTCAAGT -3'	60
		RP	5'- GATGGTCTGGGTTTCAGGTTGGA -3'	
PDGFB	24628	FP	5'- ATCCGCTCCTTTGATGACCTTC -3'	60
		RP	5'- GAGATGAGCTTTCCGACTCGAC -3'	
PEDF	287526	FP	5'- AAGAGTGCTTCCAGAATTGTG -3'	60
		RP	5'- CCCAGTTGTTAATCTCCTGAAGG -3'	

FP, Forward Primer; RP, Reverse Primer.

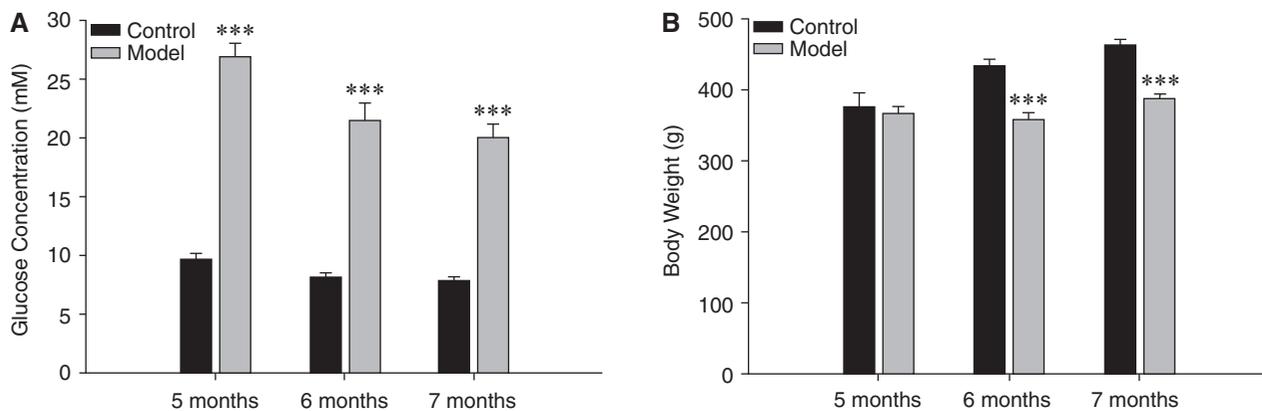


Fig. 1. Analysis of serum glucose and body weight. A: Serum glucose; B: Body weight. Data = Means \pm SEM (n = 6-12). *** P < 0.001 vs. normal Wistar rats at the same age.

in PrimeScript[®] RT Master Mix kits. Real-time PCR was performed with STEPONE Plus (Carlsbad, CA, USA) using a SYBR green premix according to the manufacturer's instructions. Relative expressions of

target genes were normalized to Actin, analyzed by $2^{-\Delta\Delta Ct}$ method and given as ratio compared to control experiments. The primer sequences used in this study are shown in Table 1.

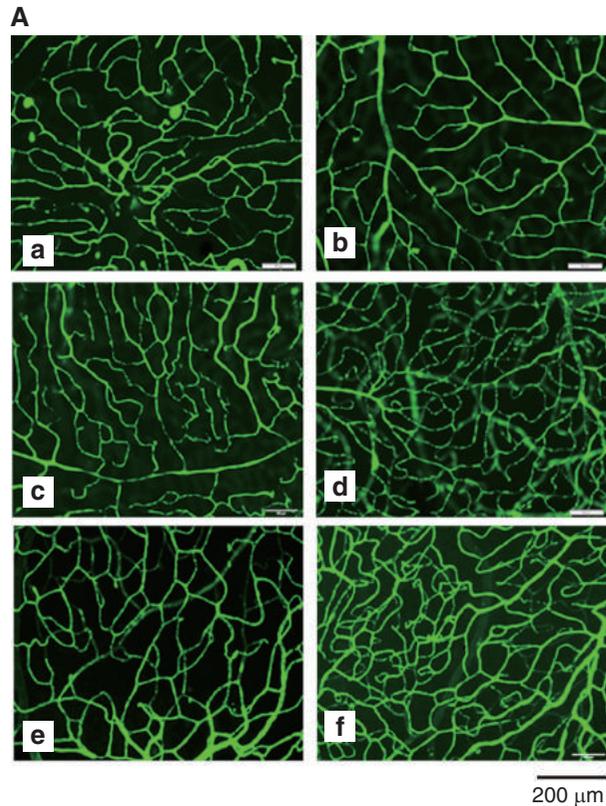


Fig. 2. Immunofluorescence staining of retinas with CD31 in normal Wistar and GK rats. (A) Typical pictures, a: Normal Wistar rats at 5 months; b: GK rats at 5 months; c: Normal Wistar rats at 6 months; d: GK rats at 6 months; e: Normal Wistar rats at 7 months; f: GK rats at 7 months; The green color represent retinal vessels. (B) Quantitative results of retinal vessels. Data = Means \pm SEM (n = 4). *** P < 0.001 vs. normal Wistar rats at the same age.

ELISA Analysis

The whole blood was gathered in a sterilization tube and centrifuged at 3500 rpm, 4°C for 15 min after stood in room temperature for 2 h, serum were collected for the further ELISA analysis according to the manufacturer's instruction.

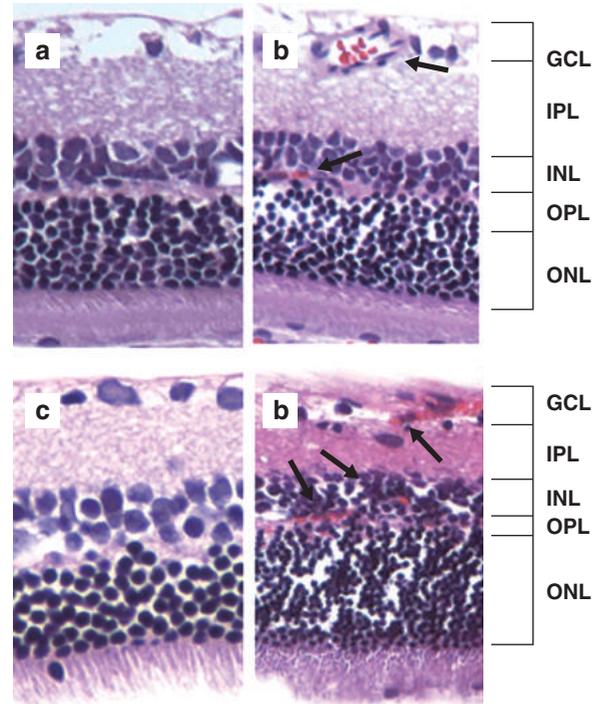


Fig. 3. Representative pictures of H and E staining of retinas in normal Wistar and GK rats a: Normal Wistar rats at month 6; b: GK rats at month 6; c: Normal Wistar rats at month 7; d: GK rats at month 7; ($\times 200$) (GCL, ganglion cell layer; IPL, inner plexiform layer; INL, inner nuclear layer; OPL, outer plexiform layer; ONL, outer nuclear layer). Arrows point out the new formed vessels.

Statistical Analysis

The results were expressed as Means \pm SEM. SPSS 18.0 was used for statistical analysis. The significance of difference between two groups was evaluated by independent-sample t -test and P < 0.05 was considered as indicating statistically significant differences.

Results

Body Weight and Blood Glucose Concentration in GK and Normal Wistar Rats

As shown in Fig. 1A, serum glucose concentration in GK rats at 5, 6 and 7 months was obviously higher than age-matched normal Wistar rats (P < 0.001). From Fig. 1B, we can see that the body weight of GK rats at 6 and 7 months were lower than age-matched normal Wistar rats (P < 0.001).

Immunofluorescence Staining of Retinal Vessels

CD31, also known as platelet endothelial cell adhesion molecule (PECAM-1), is generally used to

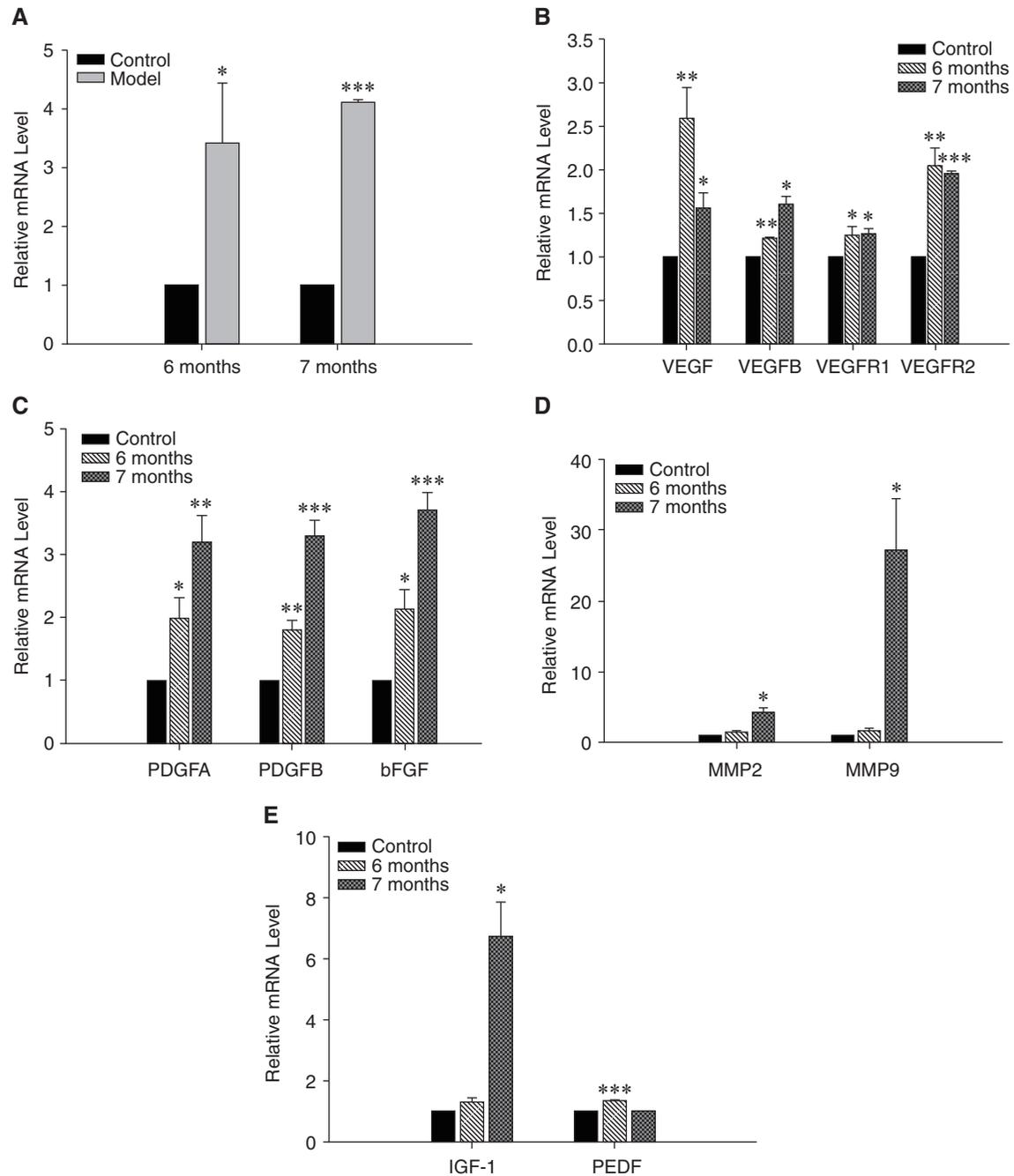


Fig. 4. Retinal mRNA expressions of some angiogenesis-related signals. (A) HIF-1 α ; (B) VEGF, VEGFB, VEGFR1 and VEGFR2; (C) PDGFA, PDGFB and bFGF; (D) MMP2 and MMP9; (E) IGF-1 and PEDF. Data = Means \pm SEM (n = 6-10). * P < 0.05, ** P < 0.01, *** P < 0.001 vs. normal Wistar rats at the same age.

identify endothelial cells, which represents the presence of vessels. Fig. 2A showed that there were more CD31 staining vessels in GK rats at 6 and 7 months (d, f) than in age-matched normal Wistar rats (c, e). However, there was no obviously increased CD31 staining vessels in GK rats at 5 months as compared to control rats (a, b). Further, after counting the vessel number, we found that retinal vessels were increased in GK rats at both 6 and 7 months as compared with

normal Wistar rats at the same age (P < 0.001).

Morphological Changes in Retina

Fig. 3 showed the morphological changes in retinas between GK and normal Wistar rats at 6 and 7 months, and there were increased vessels in ganglion cell layer (GCL), inner nuclear layer (INL) and outer nuclear layer (OPL) in GK rats at 6 and 7 months as

compared with age-matched normal Wistar rats.

Retinal mRNA Expression of Some Angiogenesis-Related Signals

Next, we analyzed the retinal mRNA expression of some angiogenesis-related genes in GK and normal Wistar rats. Fig. 4A showed that the expression of HIF-1 α , the nuclear transcription factor of VEGF, was increased in GK rats at both 6 and 7 months ($P < 0.05$, $P < 0.001$). The expression of VEGF, VEGFB and their receptors VEGFR1, VEGFR2 was all increased simultaneously in GK rats at both 6 and 7 months (Fig. 4B) ($P < 0.05$, $P < 0.01$, $P < 0.001$). The expression of other pro-angiogenic factors such as PDGFA, PDGFB and bFGF was all increased in GK rats at both 6 and 7 months (Fig. 4C) ($P < 0.05$, $P < 0.01$, $P < 0.001$). As compared with age-matched normal Wistar rats, the expression of MMP2 and MMP9 was both significantly increased in GK rats at 7 months (Fig. 4D) ($P < 0.05$), but not at 6 months. Fig. 4E showed that the expression of IGF-1 was increased in GK rats at 7 months ($P < 0.05$), but not at 6 months. The expression of PEDF was weakly increased in GK rats at 6 months ($P < 0.001$), but such increase was disappeared in GK rats at 7 months (Fig. 4E).

Serum Content of Some Angiogenesis-Related Signals

As shown in Figs. 5A-C, serum VEGF, bFGF and PDGFA contents were obviously increased in GK rats at both 6 and 7 months as compared with age-matched normal Wistar rats ($P < 0.01$, $P < 0.001$). Fig. 5D showed that serum PDGFB content was increased only in GK rats at 7 months but not 6 months ($P < 0.05$). As shown in Figs. 5E-F, serum MMP2 and MMP9 contents were increased in GK rats at both 6 and 7 months as compared with age-matched normal Wistar rats ($P < 0.001$). Serum IGF-1 content was also increased in GK rats at both 6 and 7 months (Fig. 5G) ($P < 0.01$). Fig. 5H showed that serum PEDF content was increased in GK rats at both 6 and 7 months ($P < 0.01$, $P < 0.001$).

Discussion

GK rats, the spontaneous non-obese type 2 diabetic rats with early and relatively stable hyperglycemia, hyperinsulinemia and insulin resistance, were obtained by selectively inbreeding of normal Wistar rats with the highest sugar tolerance over many generations by Goto and his collaborators in Japan in 1973 (17). Our results showed that the body weight of GK rats at both 6 and 7 months was obviously lower than age-matched normal Wistar rats, while the blood glucose level of GK rats at 5, 6 and 7 months was

stably higher than age-matched normal Wistar rats. Those results demonstrate that GK rat is an appropriate animal model for further studying of DR, which is a common and serious complication of type 2 diabetes.

Neovascularization, the key pathological process involved in PDR, is characterized by the growth of abnormal blood vessels, which may lead to various complications like vitreous hemorrhage, fibrovascular tissue formation, traction retinal detachments, and finally irreversible vision loss (1). There are some reports about the reduced retinal blood flow, retinal inflammation, and altered endothelial/pericyte ratio in GK rats (3, 27, 30), while there is still no research about the development of retinal angiogenesis and its involved signals in GK rats. The CD31 immuno-staining results showed that the number of retinal vessels was increased in GK rats at both 6 and 7 months, but not in GK rats at 5 months. In addition, retinal histological assessment demonstrated that there were more blood vessels appeared in GCL and INL layer in GK rats at both 6 and 7 months. Those above results indicate that retinal neovascularization has occurred in GK rats at 6 and 7 months.

VEGF (also referred to as VEGFA) is recognized as the most important pro-angiogenic factor at present, which can promote endothelial cell division, proliferation, and migration, and it plays an important role in regulating pathological angiogenesis (5, 21). There is report that VEGF level was obviously higher in GK rats than normal Wistar rats from 18 to 28 week, and there was a strong expression of VEGF at optic nerve fiber layer, retinal pigment epithelium and choroid in GK rats at 28 weeks (38). Our present results showed that retinal mRNA expression of VEGF and serum VEGF content were both higher in GK rats than normal Wistar rats at 6 and 7 months. VEGFR1 and VEGFR2 are receptors of VEGF distributed on endothelial cells, whose autophosphorylation will initiate the signaling cascade and induce angiogenesis (9). Our results showed that retinal expression of VEGFR1 and VEGFR2 was increased in GK rats as compared with normal Wistar rats at both 6 and 7 months, which further enhances the signaling transduction of VEGF. All those results demonstrate the critical role of VEGF/VEGFR signal pathway in the development of PDR in GK rats. VEGFB is an isoform of VEGF, and sometimes it can inhibit VEGF-induced angiogenesis in a one-to-one stoichiometric manner, but its concrete role during pathological angiogenesis has not yet been conclusively demonstrated (4, 26, 29). There is report that DR is associated with the retinal ratio of VEGF/VEGFB, and of which VEGFB can inhibit VEGF-induced retinal angiogenesis (31). Our results demonstrated that retinal mRNA expression of VEGFB was also higher in GK rats than in normal Wistar rats at

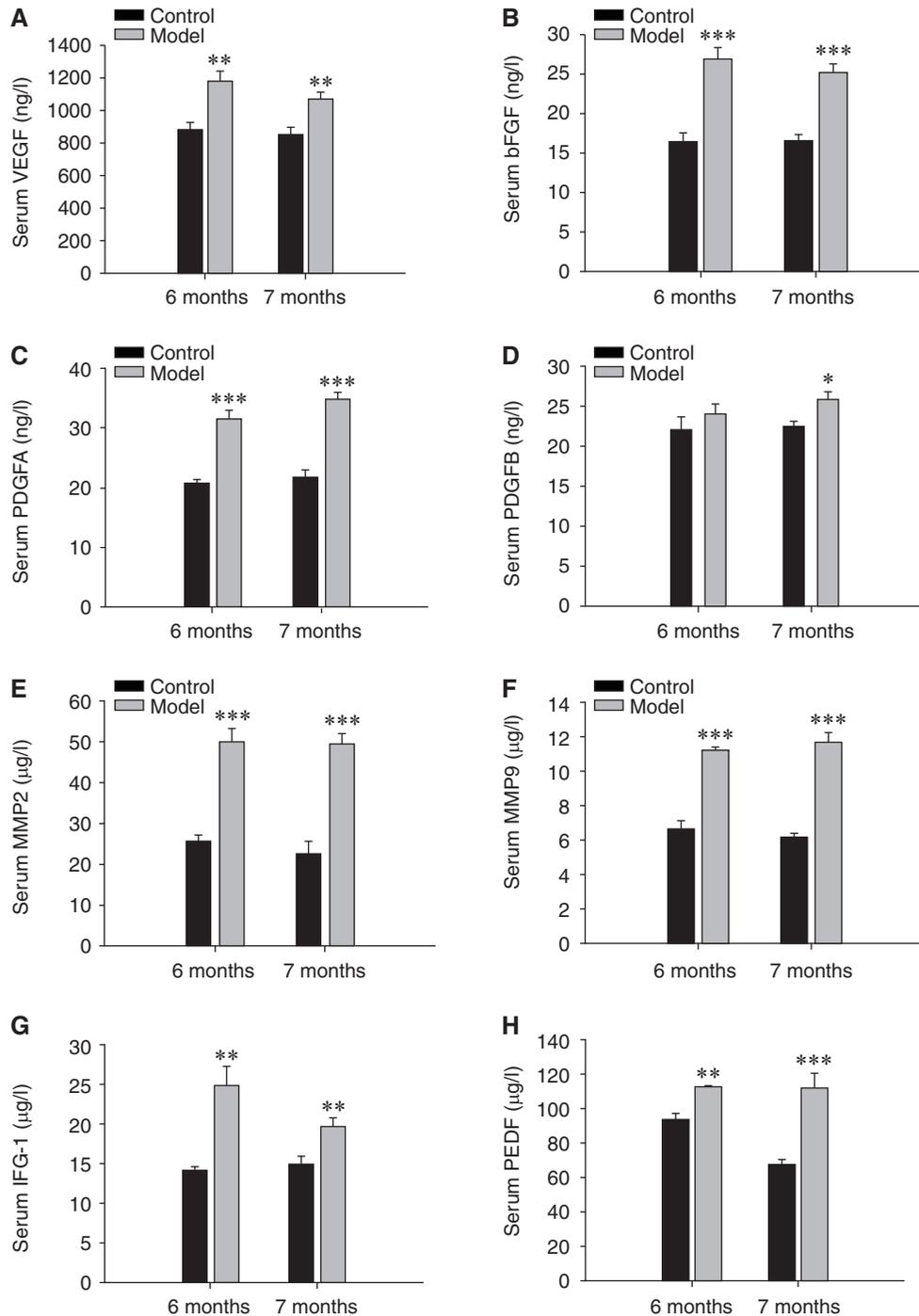


Fig. 5. Serum concentrations of some angiogenesis-related signals. (A) VEGF; (B) bFGF; (C) PDGFA; (D) PDGFB; (E) MMP2; (F) MMP9; (G) IGF-1; (H) PEDF. Data = Means \pm SEM (n = 6-10). * P < 0.05, ** P < 0.01, *** P < 0.001 vs. normal Wistar rats at the same age.

both 6 and 7 months, and whether VEGFB contributes to retinal angiogenesis or not needs further experimental evidences.

HIF-1 α is a transcription factor for dozens of target genes involving VEGF, and it is activated under hypoxia conditions and promotes VEGF transcriptional expression (46). Some studies show that HIF-1 α level

was significantly increased in the DR patients or experimental animals (25, 39, 42). Our result showed that retinal mRNA level of HIF-1 α was significantly higher in GK rats than normal Wistar rats at both 6 and 7 months, which may contribute to the increased expression of VEGF in GK rats and promote retinal neovascularization.

PDGFA and PDGFB belong to PDGF family, and they have been reported to promote the proliferation and migration of retinal pigment epithelial cells (10, 20). PDGF level was significantly increased in vitreous body in patients with DR (34). bFGF is another important growth factor involved in regulating angiogenesis, and has been reported to promote the proliferation and migration of retinal endothelial cells (7). Our results demonstrated that retinal mRNA expression and serum content of bFGF, PDGFA and PDGFB were all increased in GK rats as compared with normal Wistar rats, which suggest the involvement of those pro-angiogenic growth factors in the development of DR in GK rats.

MMP2 and MMP9 are the most widely distributed MMPs, which can degrade basement membrane, damage endothelial cell tight junction and provide the premise for the formation of new blood vessels (15). MMP2 and MMP9 was reported to be increased in diabetes and experimental animals, and drugs targeting MMP2 and MMP9 can effectively inhibit retinal angiogenesis in diabetes (6, 44). In the present study, retinal mRNA expression and serum content of MMP2 and MMP9 were both increased in GK rats as compared with normal Wistar rats, which will lead to the breakdown of blood-retinal barrier and degradation of basement membrane, and thus promote retinal neovascularization.

IGF-1 was reported to be induced expression in the pathological conditions like retinal ischemia, hypoxia during DR, and then it can promote VEGF expression and contribute to retinal angiogenesis (35, 37). Our results showed that retinal mRNA expression and serum content of IGF-1 were both increased in GK rats as compared with normal Wistar rats, which will further contribute to the development of DR in GK rats.

The balance between the pro-angiogenic and anti-angiogenic factors will be crucial for determining the progression of DR (36). PEDF, a secreted glycoprotein, is reported to prevent oxidative stress and angiogenesis, and has potential therapeutic implication in DR as the main anti-angiogenic factor (19, 43, 45). Our results demonstrated that retinal mRNA expression of PEDF was only weakly increased in GK rats at 6 months, while serum PEDF content was obviously increased in GK rats at both 6 and 7 months, which may be due to self-regulation of body in response to increased retinal angiogenesis in GK rats. Also, there were already reports that serum PEDF was increased in patients with DR (23, 28).

In conclusion, our results demonstrate that retinal neovascularization occurred at GK rats at both 6 and 7 months, accompanied by the obviously increased expression of various pro-angiogenic factors such as VEGF/VEGFR, HIF-1 α , PDGFA/B, bFGF, MMP2/9,

IGF-1 etc. Meanwhile, serum content of the main anti-angiogenic factor PEDF was also increased in GK rats. Our research will be helpful for the elucidation of the development of PDR in type 2 diabetes.

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