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 expression of hypoxia inducible factor-1α (HIF-1α), vascular endothelial growth factor (VEGF), VEGFB and its receptors (VEGFR1/2), basic fibroblast growth factor (bFGF), and platelet-derived growth factor (PDGF) A/B was increased in GK rats at both 6 and 7 months. Retinal mRNA expression of matrix metalloproteinase (MMP) 2/9 and insulin-like growth factor 1 (IGF-1) was increased at 7 months. Retinal mRNA expression of pigment epithelium-derived factor (PEDF) was increased in GK rats at 6 months. Serum content of VEGF, bFGF, PDGF, MMP2/9, IGF-1, PEDF was 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 expression of some angiogenesis related factors was 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 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 VEGF, bFGF, 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 Cξ (PKCξ) 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). 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 ± 2°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 μM), stained with haematoxylin and eosin, and examined under the microscopy (Olympus, Japan).

**Real-Time 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 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
Development of Diabetic Retinopathy in GK Rats

2ΔΔCt method and given as ratio compared to control experiments. The primer sequences used in this study are shown in Table 1.

**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.

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

<table>
<thead>
<tr>
<th>Target</th>
<th>Gene ID</th>
<th>Primer</th>
<th>Sequence</th>
<th>Annealing Temperature (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>GAPDH</td>
<td>24383</td>
<td>FP, RP</td>
<td>5’- TCCACCACCTGTGCTGTA -3’</td>
<td>60</td>
</tr>
<tr>
<td>VEGF</td>
<td>83785</td>
<td>FP, RP</td>
<td>5’- ACCACAGTCCATGCCATCAC -3’</td>
<td>60</td>
</tr>
<tr>
<td>VEGFB</td>
<td>89811</td>
<td>FP, RP</td>
<td>5’- CCCACAGGATTTTCTTGTCTT -3’</td>
<td>60</td>
</tr>
<tr>
<td>VEGFR1</td>
<td>54251</td>
<td>FP, RP</td>
<td>5’- CCTGATGGGCAAAGAATAACAT -3’</td>
<td>60</td>
</tr>
<tr>
<td>VEGFR2</td>
<td>25589</td>
<td>FP, RP</td>
<td>5’- TGAGGTAAGAGATGGGTAGG -3’</td>
<td>60</td>
</tr>
<tr>
<td>HIF-1α</td>
<td>29560</td>
<td>FP, RP</td>
<td>5’- TAGACTTGAAATGCTGCTCCC -3’</td>
<td>60</td>
</tr>
<tr>
<td>MMP2</td>
<td>81686</td>
<td>FP, RP</td>
<td>5’- AAAAGATGATGCGCTGAGG -3’</td>
<td>60</td>
</tr>
<tr>
<td>MMP9</td>
<td>81687</td>
<td>FP, RP</td>
<td>5’- GCAAAACCTGCTATTCTCATT -3’</td>
<td>60</td>
</tr>
<tr>
<td>bFGF</td>
<td>54250</td>
<td>FP, RP</td>
<td>5’- ACCTGCTATAGGAAGATGGAC -3’</td>
<td>60</td>
</tr>
<tr>
<td>IGF-1</td>
<td>24482</td>
<td>FP, RP</td>
<td>5’- GATTTTATTGCCCAGTTGGTCTTCC -3’</td>
<td>60</td>
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<tr>
<td>PDGFA</td>
<td>25266</td>
<td>FP, RP</td>
<td>5’- CTGGACAACACAGCGCTCAAGT -3’</td>
<td>60</td>
</tr>
<tr>
<td>PDGFB</td>
<td>24628</td>
<td>FP, RP</td>
<td>5’- CAGATGAGCACTTCCGACTGCAC -3’</td>
<td>60</td>
</tr>
<tr>
<td>PEDF</td>
<td>287526</td>
<td>FP, RP</td>
<td>5’- CCCAGCTGTAATCTCCTCAGG -3’</td>
<td>60</td>
</tr>
</tbody>
</table>

FP, Forward Primer; RP, Reverse Primer.

![Graph A](image1.png)

![Graph B](image2.png)

**Fig. 1.** Analysis of serum glucose and body weight. A: Serum glucose; B: Body weight. Data = Means ± SEM (n = 6-12). ***P < 0.001 vs. normal Wistar rats at the same age.
The results were expressed as Means ± 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.
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 GCL (ganglion cell layer), INL (inner nuclear layer) and OPL (outer nuclear layer) 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$\alpha$, the nuclear transcription factor of VEGF, was increased in GK rats at both 6 and 7 months ($P < 0.05$, $P < 0.001$) vs. normal Wistar rats at the same age.
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 both 6 and 7 months, and whether VEGFB contributes to retinal angiogenesis or not needs further experimental evidences.

HIF-1α 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-1α level was significantly increased in the DR patients or experimental animals (25, 39, 42). Our results showed that retinal mRNA level of HIF-1α 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 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 antiangiogenic 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-1α, PDGFα/B, bFGF, MMP2/9, IGF-1 etc. Meanwhile, serum content of the main antiangiogenic 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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References

Development of Diabetic Retinopathy in GK Rats


