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# Ocular vascular endothelial growth factor levels in diabetic rats are elevated before observable retinal proliferative changes

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**Summary** Vascular endothelial growth factor (VEGF) is a potent angiogenic factor. VEGF levels in ocular tissue of 6-, 12-, 18- and 28-week-old Goto-Kakizaki (GK) rats, a well-known model of non-insulin-dependent diabetes, were evaluated by highly sensitive ELISA. VEGF concentrations in the GK rat as well as in non-diabetic Wistar rat significantly decreased from the age of 6 weeks to 18 weeks. However, although VEGF concentrations in the Wistar rat continued to fall significantly from 18 to 28 weeks of age, the levels were maintained between 18 and 28 weeks of age in GK rats. Levels were significantly different between the GK and Wistar rats at 28 weeks of age. Results of immunohistochemical studies of the eyes of Wistar and GK rats at 28 weeks of age suggest diffuse distribution of this cytokine in cells of neural origin. Weak to moderate VEGF immunoreactivity was exhibited mainly in the ganglion cell layer, inner plexiform layer and inner/outer nuclear layers in rats with and without diabetes. However, in the retinal optic nerve fiber layer, retinal pigment epithelium and choroid, strong VEGF immunoreactivity was noted only in the GK rat. In conclusion, increased VEGF production in certain ocular tissue, similar to that in humans, is observed quite early, at least before the appearance of observable retinal changes in the diabetic GK rat. This also suggests that the GK rat can be used as a model of initial or latent phase diabetic retinopathy. [Diabetologia (1997) 40: 726–730]

**Keywords** Vascular endothelial growth factor, vascular permeability factor, Goto-Kakisaki rat, diabetic retinopathy, ELISA, immunohistochemistry.

Vascular endothelial growth factor (VEGF) (also known as vascular permeability factor) is related to platelet-derived growth factor in structure and is the strongest angiogenic cytokine known and a potent enhancer of vascular permeability. It is about 50000 times more potent than histamine. Four different isoforms, consisting of 121, 165, 189, and 206 amino acids, respectively, are generated from a single gene by alternative splicing [1]. Recent studies provide evidence of its close association with the pathophysiology of diabetic proliferative retinopathy as well as diabetic rubeotic glaucoma [2]. VEGF was considered to be produced and secreted locally in hypoxic retina by obstruction of retinal vessels, because tissue hypoxia is considered to be one of the factors that stimulates VEGF production [1]. However, as we and other investigators have reported recently, other factors, such as progesterone [3] or acute decrease in glucose concentrations [4], can be considered to stimulate VEGF production and may contribute to the deterioration of diabetic retinopathy in certain situations such as pregnancy or a rapid correction of glycaemic control. We have also reported in vitro results that exposure to a high glucose environment resulted in VEGF being up-regulation in cultured

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*Abbreviations:* VEGF, Vascular endothelial growth factor; GK rat, Goto-Kakisaki rat; NIDDM, non-insulin-dependent diabetes mellitus; PBS, phosphate-buffered saline.

retinal cells [4], showing for the first time that a high glucose environment itself up-regulates VEGF production.

The Goto-Kakisaki (GK) rat is a widely used model of spontaneous non-insulin-dependent diabetes mellitus (NIDDM) without obvious obesity. Although the diabetic state is moderate and remains stable as these animals age, glucose intolerance develops early and significant hyperglycaemia appears at around 4 weeks of age [5]. Proteinuria and decreases in nerve conducting velocity are observed, as well as pathological changes in the glomerulus and nerve tissue that are similar to those in human diabetic nephropathy and neuropathy. However, apparent retinopathy with typical proliferative lesions are not observed in these animals as they are in other rodent models of diabetes.

To clarify whether VEGF can also be induced in vivo in ocular tissue in a continuously high glucose environment, the VEGF protein level in the ocular tissue of the GK rat was examined and compared with that in control Wistar rats using our newly improved highly sensitive ELISA [6]. Furthermore, an immunohistochemical study was performed to determine localization of VEGF immunoreactivity in the ocular tissue in GK and Wistar rats.

## Materials and methods

*Tissue preparation.* Male GK rats of 6, 12, 18 and 28 weeks of age (Takeda Pharmaceutical Research Laboratories, Osaka, Japan; $n = 7$ for each age group), together with age- and weight-matched non-diabetic male Wistar rats (SLC, Hamamatsu, Japan; $n = 7$ for each age group) were deeply anaesthetized and killed by a lethal dose of pentobarbital sodium (Nembutal, Abbott Laboratories, Ill., USA) followed by cardiac perfusion of phosphate-buffered saline (PBS), and both eyes were immediately enucleated. After immersion in PBS, the diameter of each enucleated eyeball was measured laterally across the front of the eyeball using dial gauge calipers (Mitsutoyo, Utsunomiya, Japan) calibrated with 0.01 mm gradations. After volume was calculated, each eye was completely homogenized with a volume of Tris-HCl buffer (pH 7.0) 10 times that of the eye using a microhomogenizer followed by centrifugation at 4 °C for 10 min/15 000 rev/min. The supernate was frozen at –70 °C until the assay. All procedures were in accordance with The Principles of Laboratory Animal Care (NIH).

*VEGF protein assay.* VEGF concentration of the samples was determined by our previously described highly sensitive chemiluminescence ELISA [6] with slight modification. An anti-VEGF polyclonal antibody was prepared from rabbit serum immunized with recombinant human $VEGF_{121}$-glutathione-S-transferase fusion protein. Microtitre plates were coated with 10 mg/ml of the anti-VEGF antibody in 0.1 mol/l NaCl and 0.025 mol/l carbonate buffer (pH 9.0) and then blocked with 1 % bovine serum albumin, 0.2 mol/l carbonate buffer (pH 9.5), 0.1 mol/l NaCl, and 0.1 % $NaN_3$. For the assay, 100 µl of sample and serially diluted $VEGF_{121}$ were added to the wells and incubated for 1 h at 22 °C. After washing, 100 µl of alkaline phosphatase-conjugated Fab′ of the anti-VEGF antibody was added to each well and incubated for 1 h at 22 °C. After washing the wells, the enzyme reaction was carried out at 37 °C for 1 h with 3-(2′-spiroadamantane)-4 methoxy-4-(3″-phosphoryloxy)-phenyl-1,2-dioxetane disodium salt (Lumi-phos 530; Wako Pure Chemical Industries, Ltd., Osaka, Japan) as a substrate. The chemiluminescence of each well was measured by a plate luminometer, and the VEGF content was estimated from the standard curve determined from the serially diluted $VEGF_{121}$ standard. The analytical sensitivity of the assay was assessed by measuring serially diluted recombinant human $VEGF_{121}$ ranging from 1587.3 to 0 pg/ml. This immunoassay detects all known isoforms of the VEGF molecule and is not influenced by glucose concentration in the sample. The minimal detectable concentration, defined as +2SD above the zero standard, is 3 pg/ml. The intra-assay and inter-assay coefficients of variation were less than 10 % and 15 % throughout the range, respectively.

*Immunohistochemistry.* Immunohistochemical staining of sections of the whole eye from 3 male GK and 3 Wistar rats, respectively, aged 28 weeks was carried out to determine the localization of VEGF antigen in the ocular tissue using the streptavidinbiotin complex method (Histofine SAB Kit; Nichirei, Tokyo, Japan). Each rat was deeply anaesthetized and killed by a lethal dose of pentobarbital sodium followed by cardiac perfusion of 4 % paraformaldehyde (Merck, Darmstadt, Germany) 3 % sucrose PBS (pH 7.4). Eyes were enucleated and fixed in the same solution at 4 °C for 24 h before being embedded in paraffin. Each 6-mm section was deparaffinized and incubated with 0.3 % $H_2O_2$ methanol followed by incubation with 10 % normal goat serum in PBS for 10 min. Sections were then incubated with a mouse monoclonal anti-VEGF antibody, MV 303 [7] in PBS (100 µg/ml) overnight at 4 °C. Sections were incubated with biotin conjugated goat antimouse immunoglobulin for 10 min followed by washings in PBS for 10 min. Sections were then incubated with peroxidase conjugated streptavidin solution for 5 min followed by washing in PBS for 5 min. They were then stained with diaminobenzidine (Aldrich, Milwaukee, Wis., USA) solution followed by washing in tap water, then counterstained with haematoxylin and sealed by mounting media. The VEGF immunostaining was assessed using a four-grade scale (not detected, weak or slight, moderate, strong).

*Statistical analysis.* Results are expressed according to the original concentration before the tenfold dilution. Results are expressed as mean ± SD. The Mann-Whitney U-test was used for statistical analysis, and differences of $p$ less than 0.05 are considered to be significant.

## Results

VEGF concentrations in ocular tissue of control Wistar rats as well as from diabetic GK rats significantly decreased between 6 weeks (Wistar, 190.3 ± 59.1 pg/ml; GK, 197.3 ± 80.6 pg/ml) and 18 weeks (Wistar, 56.8 ± 37.6 pg/ml; GK, 86.3 ± 53.6 pg/ml) of age (Fig. 1), indicating the effects of age. However, although the VEGF concentration in Wistar rats continued to fall significantly up to 28 weeks of age (17.1 ± 7.6 pg/ml), the concentration in the GK rats maintained its level even at 28 weeks (108.7 +

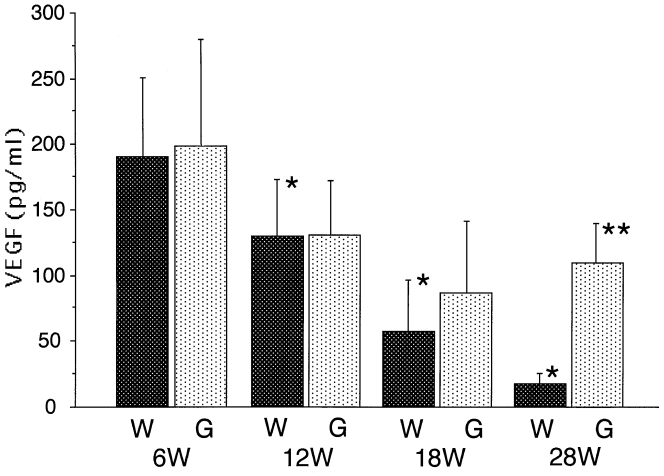


**Fig. 1.** Ocular vascular endothelial growth factor (VEGF) protein concentrations in GK (G) and non-diabetic Wistar (W) rats at 6, 12, 18 and 28 weeks of age as measured by ELISA. (Mean ± SD), $n = 7$ per age group per strain. *Significant decrease ($p < 0.05$) in concentration between time periods and between GK and Wistar rats at 28 weeks of age (**$p < 0.05$)

30.1 pg/ml) of age. As a result, there was a significant difference between the groups at 28 weeks of age ($p < 0.01$).

The results of immunostaining in Wistar and GK rats showed diffuse distribution of this cytokine in cells of neural origin (Fig. 2). Weak to moderate VEGF immunoreactivity was exhibited mainly in the ganglion cell layer (moderate), inner plexiform layer (slight) and inner/outer nuclear layers (slight) in rats with and without diabetes. Only a slight or no immunoreactivity was seen in photoreceptors in the retina of either rat. However, in the optic nerve fiber layer, retinal pigment epithelium and choroid, strong VEGF immunoreactivity was noted only in the GK rat.

## Discussion

The reduction in ocular VEGF concentrations observed in both Wistar and GK rats with age suggests that VEGF contributes to the physiological development of retinal vasculature in young animals. However, in the diabetic GK rat, the ocular VEGF level did not decrease after 18 weeks of age and stayed at the same level at least until 28 weeks of age, unlike findings for the control Wistar rats. We have recently reported that 10 days of exposure to high glucose media, although not to media with increased osmolarity, resulted in VEGF up-regulation in cultured retinal pigment epithelial cells [4]. This suggests that a continuous exposure to a high-glucose environment is required for VEGF up-regulation. As hyperglycaemia appeared as early as 4 weeks of age in the GK rat [5], it is speculated that a hyperglycaemic environment also stimulates upregulation of VEGF in vivo. Although our previous work [4] showed that only 10 days were necessary for cultured retinal pigment epithelial cells to significantly up-regulate VEGF production, the reason why more than 18 weeks was required for the difference in ocular VEGF levels between GK and Wistar rats to become significant cannot be deduced from the present study. A possible explanation may be the relatively mild diabetic status of the GK rats, which have serum glucose levels of approximately 10 mmol/l. (It was demonstrated in our previous study [4] that the retinal pigmented epithelial cells exposed to 16.5 mmol/l glucose produced a significantly increased amount of VEGF, whereas such cells exposed to 11 mmol/l glucose did not.) Furthermore, there remains the possibility that retinal cells other than retinal pigment epithelial cells require a longer exposure to a high glucose environment to up-regulate VEGF production.

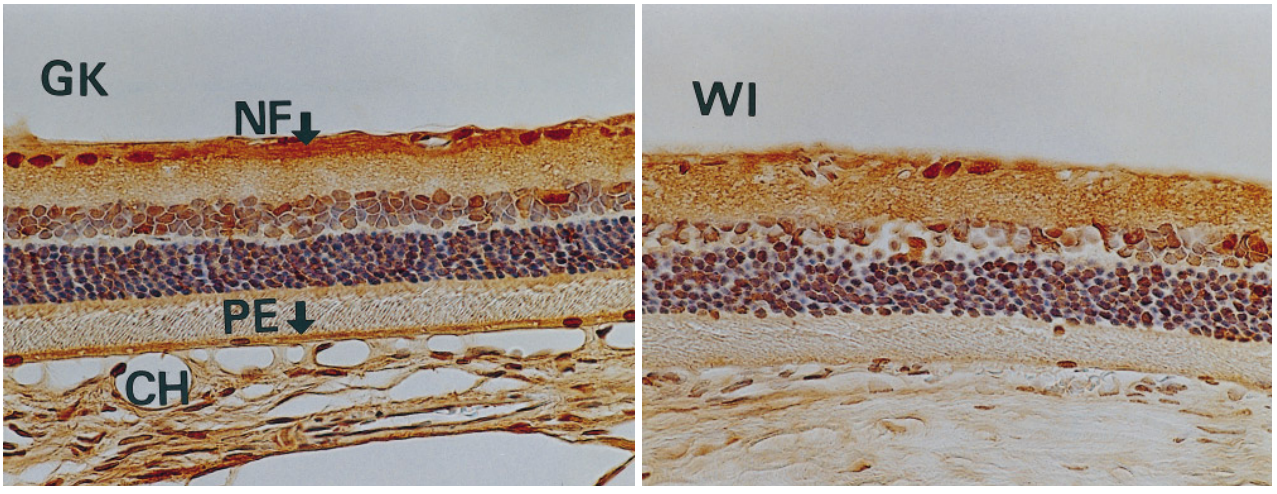


**Fig. 2.** Comparison of retina and choroid sections in the GK rat *(GK)* and non-diabetic Wistar rat *(WI)* at 28 weeks of age incubated with anti-vascular endothelial growth factor (VEGF) antibody. Stronger VEGF immunoreactivity was noted in the GK rat compared to the Wistar rat in the retinal optic nerve fiber layer *(NF)*, retinal pigment epithelium *(PE)* and choroid *(CH)*. (Diaminobenzidine reaction counterstained with haematoxylin, original magnification × 100)

The only previously reported ocular abnormality in the diabetic GK rat is a significant prolongation of retinal mean circulation time accompanied by a significant reduction in retinal blood flow at an early age before observable retinopathy developed [8]. To our knowledge, none of the other disorders that may be expected to occur, including proliferative lesions, has been observed in other diabetic model rats. VEGF was considered to be mainly up-regulated by tissue hypoxia induced by retinal vessel obstruction in the relatively late stages of diabetic retinopathy in the presence of background or preproliferative retinopathy. However, our results in the diabetic rat model suggest that the ocular VEGF level becomes elevated from very early stages of diabetes before the appearance of observable background or preproliferative retinal lesions. Moderate but continuous low perfusion and hypoxia of peripheral retinal tissue induced from reduction in retinal blood flow [8] may also contribute to the VEGF up-regulation in the early stages of the disease as well as the continuous hyperglycaemic environment in diabetes. It can also be speculated that VEGF is closely associated not only with the retinal neovascular (proliferative) lesions seen in the late stages of retinopathy, but also with the retinal exudative lesions frequently seen in the very early stages of retinopathy because of its ability to increase vascular permeability.

Although anti-human VEGF antibody was used in our assay, the measurements can be used, at least, for comparison between different groups of rats because of extremely high homogeneity in its amino acid sequence among VEGF in different species [1]. That the GK rat does not develop proliferative retinal lesions even with increased ocular VEGF levels may be due to an imbalance between angiogenic stimulators and their inhibitors, suggesting the existence of angiogenic inhibitors able to resist or neutralize the action of VEGF, particularly in early stages of diabetes.

VEGF expression determined by in situ hybridization in enucleated human eyes with rubeotic glaucoma complicated with end-stage diabetic retinopathy was reported to be upregulated mainly in the neurosensory retina [9], which was quite similar to our results in the rat model. At the same time, they noted that upregulated VEGF has already been detected even in relatively early stages of proliferative retinopathy, but because they used human tissue, it could not be determined whether its upregulation could be observed in earlier stages, such as background retinopathy. However, because we used an animal model, such information could be obtained.

Only a slight VEGF immunoreactivity was observed in retinal pigment epithelial cells in control Wistar rats, whereas significant increases were noted in the diabetic GK rat. This confirmed our previous in vitro results that retinal pigment epithelial cells have the capacity to secrete VEGF in its basic state and also to react with a high glucose environment [4]. The role of the retinal pigment epithelium in the pathophysiology of diabetic retinopathy has been overlooked because it is situated in the deepest layer of the retina. However, the retinal pigment epithelium is known to form an outer blood-retinal barrier, and its breakdown, resulting in the alteration of membrane permeability, is known to be the earliest pathological change in the diabetic retina. Retinal pigment epithelial cell-derived VEGF may also be involved in this process through autocrine pathways because of its potent action of enhancing vascular permeability.

Another large difference in VEGF immunoreactivity between diabetic and non-diabetic rats was also found in the choroid. In the diabetic GK rats, strong VEGF immunoreactivity was also associated with the choroid, whereas only weak or no choroidal VEGF distribution was observed in control Wistar rats. Recently reported immunohistochemical VEGF localization on postmortem human eyes with and without diabetes showed that VEGF immunoreactivity in the choroid was almost exclusively found in patients with diabetes [10]. The implication(s) of choroid derived VEGF in the pathophysiology of diabetic retinopathy and other ocular complications are not well understood.

In conclusion, increased VEGF production in diabetic GK rat particular ocular tissue, which is similar to that in the human, is observed at a very early stage, at least before or without the appearance of observable retinopathy. It is also suggested that the GK rat can be used as a model of early stage or latent phase diabetic retinopathy.

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