Effect of Insulin on the Expression of Intraocular Vascular Endothelial Growth Factor in Diabetic Rats

Hsi-Kung Kuo[1,2], MD; Pei-Chang Wu[1,2], MD; Chien-Neng Kuo[1], MD; Yi-Hao Chen[1], MD

Background: To investigate the long-term effect of insulin on vascular endothelial growth factor (VEGF) of streptozotocin (STZ)-induced diabetic rats.

Methods: Male Sprague-Dawley rats received intraperitoneal injections of STZ (60 mg/kg) to induce diabetes. The diabetic rats were divided into two groups: the poorly controlled diabetic group (4 U of Ultratard® week) and the insulin-controlled group (2-8 U of Ultratard® per day according to blood glucose level). The animals were sacrificed 4 months after diabetes induction. The intraocular fluids of four eyes from two rats were pooled together as one sample. VEGF was checked using an enzyme-linked immunosorbent assay (ELISA) kit.

Results: There were eight rats in the poorly controlled diabetic group, 13 in the insulin-controlled group and 10 in the healthy group. The concentration of VEGF ranged from 70.72-164.89 pg/ml (mean, 99.60 ± 31.37 pg/ml) in the poorly controlled diabetic group, 65.17-124.33 pg/ml (mean, 79.05 ± 21.50 pg/ml) in the insulin-controlled group and 50.6-67.6 pg/ml (mean, 58.07 ± 6.49 pg/ml) in the healthy group. There were statistical differences between groups (ANOVA, $p < 0.001$). The mean difference between the poorly controlled diabetic group and the insulin-controlled group was 20.55 ± 9.61 pg/ml ($p = 0.041$).

Conclusion: The concentrations of VEGF in the two diabetic rat groups were higher than that in the healthy rat group. Insulin control reduced the rise of VEGF.

(Chang Gung Med J 2006;29:555-60)

Key words: diabetic retinopathy, insulin, streptozotocin, vascular endothelial growth factor (VEGF).

Diabetic retinopathy, a leading cause of blindness, is still an important research issue. Vascular endothelial growth factor (VEGF) is a potent endothelial-selective angiogenic factor, and is essential to a number of physiological and pathological neovascular events.[1] It is temporally and spatially correlated with ocular angiogenesis in a primate model of retinal vein occlusion.[2] In diabetic retinopathy, in addition to stimulating new vessel growth during the proliferative stage of the disease, VEGF acts as a permeability factor in early diabetic retinopathy.[3] VEGF is up-regulated in early and proliferative diabetic retinopathy.[4,5] Its high level in the aqueous humor is a significant risk factor for the postoperative exacerbation of macular edema and rubeotic glaucoma.[1,6]

From the [1]Department of Ophthalmology, Chang Gung Memorial Hospital, Kaohsiung; [2]College of Medicine, Chang Gung University, Taoyuan.

Received: Mar. 3, 2006; Accepted: Jun. 1, 2006

Correspondence to: Dr. Hsi-Kung Kuo, MD, Department of Ophthalmology, Chang Gung Memorial Hospital. 123, Dabi Road, Niaosung Shiang, Kaohsiung, Taiwan 833, R.O.C. Tel.: 886-7-7317123 ext. 2801; Fax: 886-7-7318762; E-mail: d2767@cgmh.org.tw

The discovery of insulin revolutionized the treatment of diabetes and markedly extended the lives of people with this disease. Although, there was debate about the relationship of blood glucose levels and diabetic retinopathy, the Diabetes Control and Complications Trial (DCCT) demonstrated that good control of blood glucose with insulin was the most important factor in preventing or slowing the progression of diabetic retinopathy.[7] However, at the 6- and 12-month visits, a small diverse effect of intensive treatment ("early worsening") was noted. In the laboratory, insulin increased the expression of VEGF in vitro and exacerbated diabetic blood-retinal barrier breakdown in short-term studies *in vivo*.[8-12] The long-term relationship between insulin, VEGF and diabetic retinopathy is not clear. In this study, we investigated the influence of insulin on the expression of VEGF in the ocular fluids of rats 4 months after diabetes induction.

METHODS

Animals and experimental diabetes

Thirty male Sprague-Dawley rats, aged 7-8 weeks, weighing 280-360 g, were used. All animal experiments followed the Association for Research in Vision and Ophthalmology (ARVO) Statement for the Use of Animals in Ophthalmic and Vision Research. Diabetes was induced by an intraperitoneal injection of streptozotocin (Sigma-Aldrich, St. Louis, MO, USA) (60 mg/kg) in 0.05 M sodium citrate, pH 4.5. The animals were fed standard laboratory rat chow and water ad libitum. Blood glucose was determined in tail blood using a blood glucose analyzer (LifeScan, Milpitas, CA, USA) after 6 hours of fasting. Rats were considered to be diabetic when their fasting blood glucose concentrations were > 200 mg/dL. Ten normal rats were treated as the healthy control group.

Administration of insulin

Diabetic rats were treated with long-acting insulin Ultratard® HM (Novo Nordisk, Copenhagen, Denmark) 1 week after becoming diabetic and were then randomly assigned to one of two groups (15 rats/group), one poorly controlled and the other controlled with insulin by a traditional method. Group 1 rats (poorly controlled diabetic group) were given insulin 4 units once per week (to avoid a high mortality rate), while group 2 rats (insulin-controlled group) were given different doses of insulin every day according to their blood glucose levels. Blood glucose was checked twice per week. If the blood glucose concentration was 100-200, 200-300, 300-400 or > 400 mg/dL, diabetic rats were given 2, 4, 6 or 8 units of insulin per day, respectively. This protocol was based on the authors' past experience. Body weight and ocular status were checked daily. The animals were sacrificed 4 months after induction of diabetes.

Measurement of VEGF

After sacrifice, the eyeballs were enucleated immediately. They were bisected and the intraocular fluids were collected for the measurement of VEGF. As the volume of a single specimen was too small, the intraocular fluids of four eyes from two rats were pooled together as one sample, except for a single rat. For VEGF measurement, 50 μl of undiluted intraocular fluid was used. For the single rat, the intraocular fluid was double diluted and then 50 μl was used for measurement. VEGF was measured using an enzyme-linked immunosorbent assay (ELISA) kit (Quantikine, R&D Systems, Minneapolis, MN, USA) according to the manufacturer's instructions. This assay, employing an affinity purified polyclonal antibody, recognizes both 164 and 120 amino acid residue forms of mouse VEGF.

Statistical analyses

All results are expressed as means (±SD). Differences in body weights and blood glucose levels were compared using Student's *t* test. Differences in VEGF between groups were compared by one-way analysis of variance (ANOVA) followed by the post hoc test (least significant difference (LSD) test) for comparison of pairs of groups. Differences were considered statistically significant when the *p* values were less than 0.05.

RESULTS

Experimental diabetes

Nine rats died before completion of the 4-month diabetic period (most within the first month) and were, thus, excluded from the analyses. There were eight rats in the poorly controlled diabetic group, 13 in the insulin-controlled group and 10 in the healthy

group.

Glucose control and body weight

In the poorly controlled diabetic group, the average blood glucose level was 380.43 ± 96.45 mg/dL. In the insulin-controlled group, the average blood sugar was 364.66 ± 99.23 mg/dL. The difference between these 2 groups was not statistically significant (Student's *t* test, $p = 0.1295$). In the poorly controlled diabetic group, the average initial body weight was 323.25 ± 30.67 g and the final body weight was 313.5 ± 40.86 g (a loss of 2.45%). In the insulin-controlled group, the average initial body weight was 330.31 ± 27.57 g and the final body weight was 429.46 ± 46.87 g (a gain of 30.68%). The change in body weight between the two groups was statistically significant (Student's *t* test, $p = 0.00134$) (Table 1). The insulin-controlled group of rats had better growth.

VEGF concentration

The concentration of VEGF was 70.72-164.89 pg/ml (mean, 99.60 ± 31.37 pg/ml) for the poorly controlled diabetic group, 65.17-124.33 pg/ml (mean, 79.05 ± 21.50 pg/ml) for the insulin-controlled group and 50.6-67.6 pg/ml (mean, 58.07 ± 6.49 pg/ml) for the healthy group (Fig. 1). There were statistically significant differences between the groups (ANOVA, $p < 0.001$). The comparison of pairs of groups using the LSD test showed that the mean difference between the poorly controlled diabetic group and the healthy group was 41.53 ± 10.15 pg/ml ($p < 0.001$), between the insulin-controlled group and the healthy group was 20.98 ± 9.00 pg/ml ($p = 0.027$), and between the poorly controlled diabetic group and the insulin-controlled group was 20.55 ± 9.61 pg/ml ($p = 0.041$) (Table 2). The VEGF concentrations of the two diabetic rat groups were higher than that of the healthy rat group. Insulin control can reduce the rise of VEGF and the difference in VEGF concentrations between the two diabetic groups was statistically significant.

Table 1. Blood Glucose Level and Body Weight Change in Diabetic Rats

	Insulin-controlled group	Poorly controlled diabetic group	*p* value
n	296	129	
Glucose level (mg/dL)	364.66 ± 99.23	380.43 ± 96.45	0.1295
N	13	8	
Initial BW (g)	330.31 ± 27.57	323.25 ± 30.67	
Final BW (g)	429.46 ± 46.87	313.5 ± 40.86	
BW gain	+30.68%	-2.45%	0.00134

Abbreviations: n: total number of blood glucose tests; N: number of diabetic rats; BW: body weight; BW gain: body weight difference calculated by the formula (final BW-initial BW/initial BW).

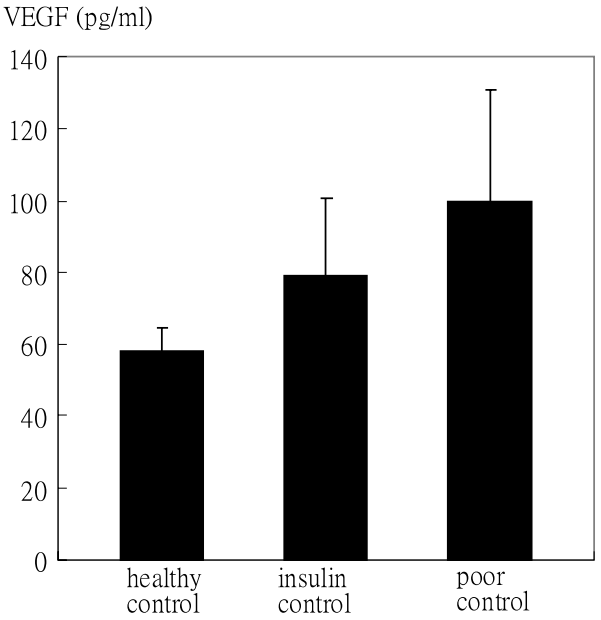

Fig. 1 Vascular Endothelial Growth Factor (VEGF) concentration in different groups.

DISCUSSION

Studies of animal diabetes show that hyperglycemia is the primary insult in the pathogenesis of

Table 2. Vascular Endothelial Growth Factor (VEGF) Concentrations in Different Groups

	Healthy group	Insulin-controlled group	Poorly controlled diabetic group
N	10	13	8
VEGF (pg/ml)	58.07 ± 6.49	79.05 ± 21.50	99.60 ± 31.37
Healthy	-	$p = 0.027$*	$p < 0.001$*
Insulin-controlled	$p = 0.027$*	-	$p = 0.041$*
Poorly controlled diabetic	$p < 0.001$*	$p = 0.041$*	-

Abbreviation: N: number of diabetic rats.
*$p < 0.05$.

diabetic retinopathy. Several metabolic perturbations, including the polyol pathway, oxidative damage and non-enzymatic glycation, may be activated secondary to hyperglycemia. These biochemical alterations may further cause alternation of vasoactive factors influencing blood and permeability. VEGF is one of the most important vasoactive factors.[3,13-16] VEGF is a potent angiogenic factor and permeability factor, and it is up-regulated in early and proliferative diabetic retinopathy.[2,4,5,17-20] The therapeutic effect of panretinal photocoagulation on proliferative diabetic retinopathy is exerted by reduction of the level of VEGF in ocular fluid.[21] VEGF could be an important monitoring index for diabetic retinopathy.

Whereas the institution of strict glycemic control retards the development and progression of retinopathy over the long term, acute intensive insulin therapy might initially result in an early deterioration of diabetic retinopathy in patients with long-standing poor glycemic control, especially if retinopathy is at or past the moderate nonproliferative stage.[7,22] Recently, insulin has been reported to increase the expression of VEGF in different cell types through multiple signaling pathways.[8,9,23] Lu et al., Qaum et al. and Poulaki et al. demonstrated that acute intensive insulin therapy markedly increased VEGF mRNA and protein levels, and blood-retinal barrier breakdown in the retinas of 8-day diabetic rats. They correlated these findings to the transient, early worsening of diabetic retinopathy.[10-12] They also showed insulin-induced VEGF expression required p38 mitogen-activated protein kinase (MAPK) and phosphatidylinositol 3-kinase (PI 3-K), whereas hyperglycemia-induced VEGF expression was hypoxia-inducible factor 1 alpha (HIF-1α)-independent and required protein kinase C (PKC) and p42/p44 MAPK.[11,12] Insulin-like growth factor-1 (IGF-1) and IGF-2 are other members of the insulin signaling family. IGF-1 has been shown to promote angiogenesis by enhancing VEGF expression.[24,25] Poulaki et al. also showed that IGF-1 participated in the pathophysiology of diabetic retinopathy by inducing retinal VEGF expression via PI-3K/protein kinase B (AKT), HIF-1α and nuclear factor kappa B (NF-κB) activation.[26] However, few animal studies have focused on the long-term effects of insulin.

For conventional (non-intensive), long-term insulin treatment, the effect of insulin on the expression of VEGF is unclear. Here, we studied non-intensive insulin treatment in the diabetic rat model. Given the insulin treatment protocols in this study, the average blood glucose of the insulin controlled group was not significantly lower than the poorly controlled diabetic group. This result might be due to free food intake, lack of daily monitoring and insufficient sugar control. However, the average body weight increase was significantly different. Insulin still improved the growth of these diabetic rats and reduced the rise of VEGF in 4-month duration diabetic rats. This finding is compatible with most clinical studies that have shown that VEGF is lower in eyes of subjects with milder diabetes. However, the different results of insulin on VEGF production in our study, and other in vitro and short-term in vivo studies, could not be reconciled in this paper and require further effort to investigate other changes in long-term diabetic rats.

REFERENCES

1. Sone H, Okuda Y, Kawakami Y, Hanatani M, Suzuki H, Kozawa T, Honmura S, Yamashita K. Vascular endothelial growth factor level in aqueous humor of diabetic patients with rubeotic glaucoma is markedly elevated. Diabetes Care 1996;19:1306-7.
2. Miller JW, Adamis AP, Shima DT, D' Amore PA, Moulton RS, O' Reilly MS, Folkman J, Dvorak HF, Brown LF, Berse B, Yeo TK, Yeo KT. Vascular endothelial growth factor/vascular permeability factor is temporally and spatially correlated with ocular angiogenesis in a primate model. Am J Pathol 1994;145:574-84.
3. Chakrabarti S, Cukiernik M, Hileeto D, Evans T, Chen S. Role of vasoactive factors in the pathogenesis of early changes in diabetic retinopathy. Diabetes Metab Res Rev 2000;16:393-407.
4. Hammes HP, Lin J, Bretzel RG, Brownlee M, Breier G. Upregulation of the vascular endothelial growth factor/vascular endothelial growth factor receptor system in experimental background diabetic retinopathy of the rat. Diabetes 1998;47:401-6.
5. Pe'er J, Folberg R, Itin A, Gnessin H, Hemo I, Keshet E. Upregulated expression of vascular endothelial growth factor in proliferative diabetic retinopathy. Br J Ophthalmol 1996;80:241-5.
6. Funatsu H, Yamashita H, Noma H, Shimizu E, Mimura T, Hori S. Prediction of macular edema exacerbation after phacoemulsification in patients with nonproliferative diabetic retinopathy. J Cataract Refract Surg 2002;28:1355-63.
7. The Diabetes Control and Complications Trial Research Group. The effect of intensive diabetes treatment on the progression of diabetic retinopathy in insulin-dependent

diabetes mellitus. Arch Ophthalmol 1995;113:36-51.
8. Bermont L, Lamielle F, Lorchel F, Fauconnet S, Esumi H, Weisz A, Adessi GL. Insulin up-regulates vascular endothelial growth factor and stabilizes its messengers in endometrial adenocarcinoma cells. J Clin Endocrinol Metab 2001;86:363-8.
9. Mick GJ, Wang X, McCormick K. White adipocyte vascular endothelial growth factor: regulation by insulin. Endocrinology 2002;143:948-53.
10. Lu M, Amano S, Miyamoto K, Garland R, Keough K, Qin W, Adamis AP. Insulin-induced vascular endothelial growth factor expression in retina. Invest Ophthalmol Vis Sci 1999;40:3281-6.
11. Qaum T, Xu Q, Joussen AM, Clemens MW, Qin W, Miyamoto K, Hassessian H, Wiegand SJ, Rudge J, Yancopoulos GD, Adamis AP. VEGF-initiated blood-retinal barrier breakdown in early diabetes. Invest Ophthalmol Vis Sci 2001;42:2408-13.
12. Poulaki V, Qin W, Joussen AM, Hurlbut P, Wiegand SJ, Rudge J, Yancopoulos GD, Adamis AP. Acute intensive insulin therapy exacerbates diabetic blood-retinal barrier breakdown via hypoxia-inducible factor-1 α and VEGF. J Clin Invest 2002;109:805-15.
13. Wu G. Nitric oxide synthesis and the effect of aminoguanidine and NG-monomethyl-L-arginine on the onset of diabetes in the spontaneously diabetic BB rat. Diabetes 1995;44:360-4.
14. Lindsay RM, Smith W, Rossiter SP, McIntyre MA, Williams BC, Baird JD. N omega-nitro-L-arginine methyl ester reduces the incidence of IDDM in BB/E rats. Diabetes 1995; 44:365-8.
15. Hammes HP, Martin S, Federlin K, Geisen K, Brownlee M. Aminoguanidine treatment inhibits the development of experimental diabetic retinopathy. Proc Natl Acad Sci USA 1991;88:11555-8.
16. Agardh E, Hultberg B, Agardh CD. Effects of inhibition of glycation and oxidative stress on the development of cataract and retinal vessel abnormalities in diabetic rats. Curr Eye Res 2000;21:543-9.
17. Aiello LP, Avery RL, Arrigg PG, Keyt BA, Jampel HD, Shah ST, Pasquale LR, Thieme H, Iwamoto MA, Park JE, Nguyen HV, Aiello LM, Ferrara N, King GL. Vascular endothelial growth factor in ocular fluid of patients with diabetic retinopathy and other retinal disorders. N Engl J Med 1994;331:1480-7.
18. Malecaze F, Clamens S, Simorre-Pinatel V, Mathis A, Chollet P, Favard C, Bayard F, Plouet J. Detection of vascular endothelial growth factor messenger RNA and vascular endothelial growth factor-like activity in proliferative diabetic retinopathy. Arch Ophthalmol 1994;112:1476-82.
19. Tanaka Y, Katoh S, Hori S, Miura M, Yamashita H. Vascular endothelial growth factor in diabetic retinopathy. Lancet 1997;349:1520.
20. Mathews MK, Merges C, McLeod DS, Lutty GA. Vascular endothelial growth factor and vascular permeability changes in human diabetic retinopathy. Invest Ophthalmol Vis Sci 1997;38:2729-41.
21. Shinoda K, Ishida S, Kawashima S, Wakabayashi T, Uchita M, Matsuzaki T, Takayama M, Shinmura K, Yamada M. Clinical factors related to the aqueous levels of vascular endothelial growth factor and hepatocyte growth factor in proliferative diabetic retinopathy. Curr Eye Res 2000;21:655-61.
22. The Diabetes Control and Complications Trial Research Group. Early worsening of diabetic retinopathy in the Diabetes Control and Complications Trial. Arch Ophthalmol 1998;116:874-86.
23. Jiang ZY, He Z, King BL, Kuroki T, Opland DM, Suzuma K, Suzuma I, Ueki K, Kulkarni RN, Kahn CR, King GL. Characterization of multiple signaling pathways of insulin in the regulation of vascular endothelial growth factor expression in vascular cells and angiogenesis. J Biol Chem 2003;278:31964-71.
24. Slomiany MG, Rosenzweig SA. IGF-1-induced VEGF and IGFBP-3 secretion correlates with increased HIF-1 alpha expression and activity in retinal pigment epithelial cell line D407. Invest Ophthalmol Vis Sci 2004;45:2838-47.
25. Rosenthal R, Wohlleben H, Malek G, Schlichting L, Thieme H, Bowes Richman C, Strauss O. Insulin-like growth factor-1 contributes to neovascularization in age-related macular degeneration. Biochem Biophys Res Commun 2004;323:1203-8.
26. Poulaki V, Joussen AM, Mitsiades N, Mitsiades CS, Iliaki EF, Adamis AP. Insulin-like growth factor-I plays a pathogenetic role in diabetic retinopathy. Am J Pathol 2004;165:457-69.

胰島素對糖尿病鼠眼內血管內皮生長因子表達的影響

郭錫恭[1,2] 吳佩昌[1,2] 郭千能[1] 陳怡豪[1]

背 景： 爲了研究長期使用胰島素對 streptozotocin 引發的糖尿病鼠眼內的血管生長因子的影響。

方 法： 在雄性大白鼠腹腔注射 streptozotocin (60 毫克／每公斤體重) 來引發糖尿病。糖尿病鼠分成兩組：控制不良組 (每週打 1 次 4 單位 Ultratard®) 和胰島素控制組 (每天依血糖濃度打 2-8 單位 Ultratard® 不等)。糖尿病鼠 4 個月後犧牲。2 隻老鼠的 4 顆眼球內的眼內液合爲一個標本。血管內皮生長因子是使用 ELISA 方法測定。

結 果： 4 個月後在控制不良組有 8 隻糖尿病鼠，胰島素控制組有 13 隻糖尿病鼠，另外再使用 10 隻健康鼠當控制組。血管內皮生長因子在上述三組的濃度依序是 70.72~164.89 (平均 99.60±31.37)，65.17~124.33 (平均 79.05±21.50) 及 50.6~67.6 (平均 58.07±6.49) pg/ml。三組間有統計學上顯著差異 (ANOVA，$p < 0.001$)。在控制不良組和胰島素控制組之間的差是 20.55±9.61 pg/ml ($p = 0.041$)，有統計學上的差異。

結 論： 在兩組糖尿病鼠眼內的血管內皮生長因子濃度比健康鼠高。胰島素的控制可以抑制血管內皮生長因子濃度的提高。

(長庚醫誌 2006;29:555-60)

關鍵語： 糖尿病視網膜病變，胰島素，streptozotocin，血管內皮生長因子。

長庚紀念醫院 高雄院區 [1]眼科；[2]長庚大學 醫學院
受文日期：民國95年3月3日；接受刊載：民國95年6月1日
通訊作者：郭錫恭醫師，長庚紀念醫院 眼科。高雄縣833鳥松鄉大埤路123號。Tel.: (07)7317123轉2801; Fax: (07)7318762; E-mail: d2767@adm.cgmh.org.tw