Increased Vascular Endothelial Growth Factor mRNA Expression in the Heart of Streptozotocin-Induced Diabetic Rats

F.C. Sasso, O. Carbonara, E. Persico, R. D'Ambrosio, L. Coppola, R. Nasti, B. Campana, S. Moschella, R. Torella, and D. Cozzolino

The aim of the present study was to evaluate vascular endothelial growth factor (VEGF), fms-like tyrosine kinase 1 (flt-1), and fetal liver kinase (flk-1) expression in the heart of experimental diabetic rats. Ten young adult male Wistar rats (5 streptozotocin [STZ]-induced diabetic rats, without insulin treatment, and 5 controls) were studied. Ninety days after the induction of diabetes, semiquantitative reverse transcription (RT)-polymerase chain reaction (PCR) coamplification of VEGF/glyceraldehyde 3-phosphate dehydrogenase (GAPDH) transcription was performed. RT-PCR was also performed for VEGF receptors flk-1 and flt-1. VEGF mRNA expression, at 234 bp, was detectable in the heart of the rats and was significantly higher in those with diabetes. Densitometric analysis of PCR products showed that VEGF mRNA levels were meanly 4.8-fold higher in STZ-induced diabetic rats than controls (VEGF/GAPDH densitometric ratio, 3.46 ± 0.20 *v* 0.74 ± 0.10, *P* < .001). No significant difference was found in flt-1 and flk-1 amplification products between STZ-induced diabetic rats and controls (flt-1/GAPDH densitometric ratio, 0.58 ± 0.01 *v* 0.64 ± 0.05, *P* > .1; flk-1/GAPDH densitometric ratio, 0.66 ± 0.10 *v* 0.7 ± 0.06, *P* > .2). The increase in VEGF mRNA expression observed in this experimental diabetic model is in contrast with the typical impairment in collateral vessels of diabetic hearts. This apparent discrepancy might be explained by a resistance of cardiac tissue to VEGF. The lack of mRNA flt-1 and flk-1 overexpression in diabetic hearts could be one of the mechanisms for this resistance.

VASCULAR ENDOTHELIAL growth factor (VEGF) is a cytokine inducing angiogenesis in vitro and in vivo.[1] Reduced tissue oxygen tension, as in ischemic regions, leads to the development of new blood vessels to satisfy the needs of the tissues, and VEGF is considered a hypoxia-inducible angiogenic factor.[2] Two high-affinity VEGF receptors have been described in vascular endothelium, fms-like tyrosine kinase 1 (flt-1) and fetal liver kinase (flk-1).[3]

Diabetic microvascular complications are considered to be influenced by the increase of angiogenic factors, such as VEGF, as a response to both ischemia and hyperglycemia.[4] The role of these factors in macrovascular complications has not been fully investigated. If large vessels are affected by atherosclerosis, as in diabetic disease, the development of collateral vessels can reduce the degree of ischemic damage. Several observations suggest that VEGF may play a significant role in this adaptive process.[5] Moreover, VEGF mRNA expression greatly increases after cardiac infarction in the human heart.[6]

Impairments in VEGF expression and action have been found in diabetes mellitus.[7,8] Whether diabetes-related heart disease is associated with altered expression of VEGF is unclear. Very recently a decreased VEGF and its receptors mRNA expression was observed in diabetic rat heart after 4 weeks of disease.[9] Our study was therefore designed to evaluate VEGF, flt-1, and flk-1 expression in the hearts of long-term experimental diabetic rats.

MATERIALS AND METHODS

Animals

Thirty young adult male Wistar rats weighing 250 to 300 g, housed in the Department of Pharmocology at the Second University of Naples, were divided into 2 groups.

Diabetes was induced in the animals of the first group by streptozotocin (STZ, Sigma, St Louis, MO, 45 mg/kg, intraperitoneal injection), while the animals of the second group (control) received the carrier (pH 5.5, aceto-acetic buffer, intraperitoneal injection). Blood samples were collected from the tail vein before, and 48 hours and 90 days after STZ administration to measure serum glucose by the glucose oxidase method (Beckman Glucose Analyzer II, Fullerton, CA). At the same times, urine samples were collected to test urine glucose (Diabur-Test 5000, Roche Diagnostics GmbH, Mannheim, Germany). The rats were defined as diabetic when blood glucose concentration exceeded 300 mg/dL and urine glucose was positive 48 hours after the STZ injection. Diabetic rats received no insulin therapy, in order to increase the damage induced by hyperglycemia. Food and water were available ad libitum to both groups.

Ninety days after diabetes induction, only 5 diabetic rats were still alive. These animals were killed together with 5 control animals. Each animal was decerebrated while under anesthesia with xilazin and ketamine. The heart was soon removed and immediately frozen in liquid nitrogen. Just before removing the heart, blood samples were drawn from the aorta to assess serum VEGF by enzyme-linked immunosorbent assay (R&D Systems, Indianapolis, IN).

The procedures were performed in accordance with the standard principles of laboratory animal care, as well as Italian Law on the protection of animals.

Reverse-Transcription Polymerase Chain Reaction Analysis

Frozen hearts were maintained in liquid nitrogen until reverse-transcription (RT) polymerase chain reaction (PCR) analysis was performed on total RNA isolated from tissues by acid guanidinium thiocyanate-phenol-chloroform extraction, according to the Chomczynski and Sacchi method.[10] First-strand complementary DNA was prepared using 200 U of reverse transcriptase (Supertranscript RT, Gibco BRL, Gaithersburg, MD), 1 μg of total RNA as template, and 10 pmol/L of random hexamers in the presence of 0.1 mmol/L dithiothreitol, 0.5 mmol/L dNTP-litium salt (Pharmacia, Milan, Italy), and 20 U of RNase inhibitor (Promega, Madison, WI). The reaction profile was 37°C for 10 minutes, followed by 42°C for 60 minutes. Semiquantitative RT-

From the Unit of Internal Medicine and Metabolism, Second University of Naples, Naples, Italy.

Submitted June 7, 2002; accepted January 14, 2003.

Address reprint requests to Ferdinando Carlo Sasso, MD, PhD, Professor of Internal Medicine, Second University of Naples, Via F. Petrarca, 64, I-80122, Napoli, Italy.

0026-0495/03/5206-0012$30.00/0
doi:10.1016/S0026-0495(03)00064-7

Table 1. Weight, Glycemia, and Serum VEGF of Study Rats

	Controls		Diabetic Animals	
	48 h	90 d	48 h	90 d
Weight (g)	270 ± 15	350 ± 27	250 ± 11	180 ± 15*
Glycemia (mg/dL)	90 ± 8	94 ± 6	365 ± 20*	381 ± 35*
Serum VEGF (pg/mL)	—	86 ± 10.8	—	114 ± 9.6*

NOTE. Values are mean ± SD.
*$P < .01$ *v* controls.

PCR coamplification of VEGF/glyceraldehyde 3-phosphate dehydrogenase (GAPDH) transcript was performed using specific primers provided by Roche Diagnostics S.p.a. (Monza, Italy): sense VEGF (5′ TTC-ATG-GAT-GTC-TAT-CAG-CG 3′), antisense VEGF (5′GCT-CAT-CTC-TCC-TAT-GTG-CT 3′); sense GAPDH (5′ CGA-TGC-TGG-CGC-TGA-GTA-C 3′), and antisense GAPDH (5′ CGT-TCA-GCT-CAG-GGA-TGA-CC 3′). After an initial denaturation step, 95°C for 2 minutes, the PCR amplification was performed using the following profiles: 94°C for 30 seconds, 56°C for 60 seconds, and 72°C for 60 seconds for 40 cycles. Twelve-microliter samples from each 50 μL PCR product were removed and analyzed by electrophoresis on ethidium bromide-coloured 1.8% agarose gel. Known weight markers (P9577, Sigma-Aldrich, Milan, Italy) were used to establish the weight of amplified DNA electrophoretic bands.

The gel images were recorded by video camera (Sony Video Camera Module CCD, Tokyo, Japan), connected to an IBM AT computer (New York, NY) with a 512-by-512 pixel array imaging board with 256 grey levels. PCR products were quantified by densitometric scanning of gel images using RFL Print (BDI, Dublin, Ireland) software. Results were then expressed as VEGF/GAPDH densitometric ratio.

PCR was also performed for VEGF receptors, using the following primers: sense flt-1 (5′ CAT-GGT-CAG-CTG-GGA-CAC-CGC-G 3′) and antisense flt-1 (5′ GAC-TCC-CTG-CAT-CAC-TAA-CAA-TAT 3′), sense flk-1 (5′ TCA-GAT-TAC-TTG-CAG-GGG-ACA-GAG-G 3′), and antisense flk-1 (5′ GGA-TCA-CCA-CAG-TTT-TGT-TCT-TGT-T 3′). The following cycle parameters were used: 94°C for 45 seconds, 62°C for 30 seconds, and 72°C for 120 seconds; the reactions were allowed to repeat for 35 cycles for flt-1 and 36 cycles for flk-1.

Statistical Analysis

Analysis of variance (ANOVA) for repeated measures was used to compare variables within one group at different times, while the Student's *t* test for unpaired samples was used to compare control and diabetic animals. VEGF, flt-1, and flk-1/GAPDH densitometric ratio values from the 2 groups were compared by the Student's *t* test for unpaired samples.

Statistical analysis was performed using SPSS 8.0 for Windows (SPSS, Inc, Chicago, IL). A P value $< .05$ was considered significant. Data in the text and tables are expressed as mean±SD.

RESULTS

Serum glucose concentrations, as well as urine glucose (data not shown), confirmed successfully developed experimental diabetes after STZ injection, while body weight was consistently decreased in the diabetic rats, as expected (Table 1). Serum VEGF levels were shown to be significantly higher in diabetic rats compared with nondiabetic rats after 90 days (Table 1).

VEGF mRNA expression, at 234 bp, as assessed by semiquantitative RT-PCR coamplification of VEGF/GAPDH transcripts, was detectable in the rat heart and was significantly higher in those with diabetes (Fig 1).

The electrophoretic migration in the region of GAPDH gene, at 470 bp, was seen in diabetic and control animals. Densitometric analysis confirmed a similar amount of GAPDH mRNA in all the hearts studied.

By densitometric analysis of PCR products, VEGF mRNA levels were meanly 4.8 fold (range, 3.4 to 6.2) higher in STZ-induced diabetic rats than controls (VEGF/GAPDH densitometric ratio, 3.46 ± 0.20 *v* 0.74 ± 0.10, $P < .001$) (Fig 2).

No difference was found between STZ-induced diabetic rats and controls in flt-1 and flk-1 amplification products at approximately 400 bp and approximately 300 bp, respectively (flt-1/GAPDH densitometric ratio, 0.58 ± 0.01 *v* 0.64 ± 0.05, $P > .1$; flk-1/GAPDH densitometric ratio, 0.66 ± 0.10 *v* 0.7 ± .06, $P > .2$).

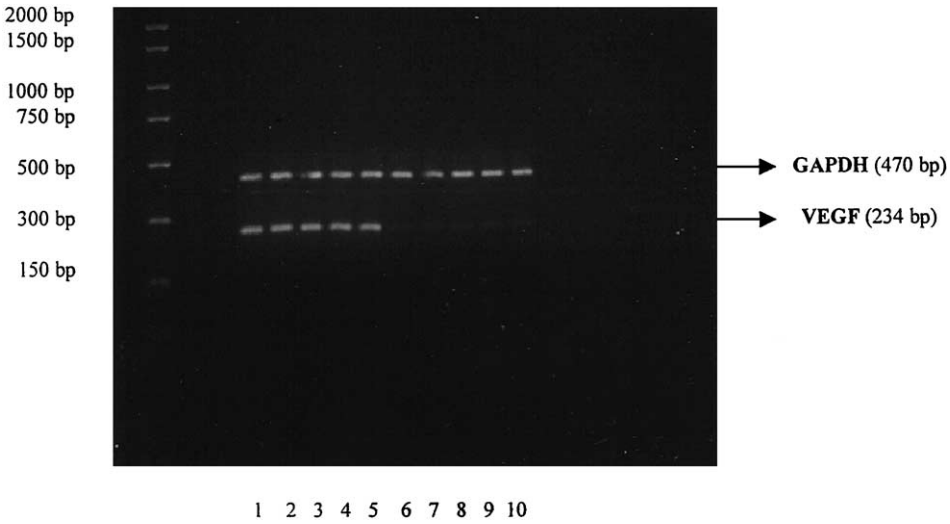

Fig 1. Analysis of VEGF mRNA expression in the heart of diabetic (lines 1-5) and control (lines 6-10) rats by electrophoresis of RT-PCR products. The RT-PCR products of 234 bp and 470 bp correspond to VEGF cDNA and GAPDH cDNA, respectively. The line on the left of the panel shows known weight markers.

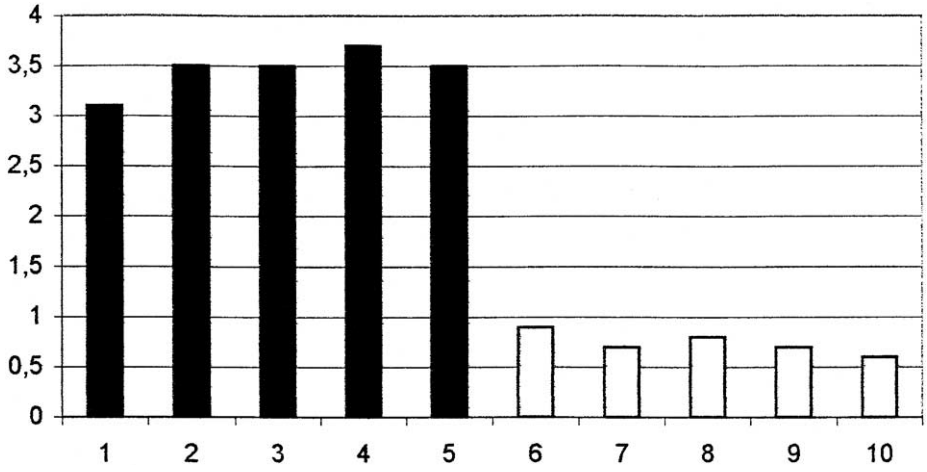

Fig 2. Quantification of heart VEGF mRNA in diabetic (■) and control (□) rats. Data are the ratio values of VEGF and GAPDH mRNA optical density.

DISCUSSION

A decreased VEGF and its receptors mRNA expression has recently been observed in diabetic rat heart after 4 weeks of disease.[9]

This is the first study to reveal an increased VEGF mRNA expression within the heart of long-term experimental diabetic rats. Upregulation of VEGF mRNA expression in the heart of diabetic rats might be secondary to the hypoxia related to the high glycosylated hemoglobin levels and the decrease in nitric oxide caused by hyperglycemia and generation of reactive oxygen species.

VEGF plays an important role in the development of collateral vessels in ischemic myocardium.[5] Structural and functional abnormalities of the coronary collateral microcirculation have been reported in clinical and experimental diabetes mellitus,[11] and this might partly explain the higher severity of myocardial ischemia and post-ischemic deficit of ventricular function in diabetic patients.[12] The increased VEGF mRNA expression in our study seems in contrast with the impairment in collateral vessels. This apparent discrepancy might be explained by a resistance of cardiac tissue to VEGF. In partial support of this hypothesis, an attenuated cellular response of monocytes to VEGF due to a downstream signal transduction defect has recently been observed in diabetic patients.[7] Thus, monocyte-dependent angiogenesis may be reduced in diabetes mellitus. Moreover, conditions of hyperglycemia in vitro reduced cellular response to VEGF,[13] while VEGF expression was upregulated under high glucose conditions in vascular smooth muscle cells.[14] If these findings are confirmed, the successful use of VEGF agonists to stimulate collateral vessel neogenesis and to reduce coronary artery disease symptoms in nondiabetic patients[15] could fail in diabetic subjects.

We detected no significant change in VEGF receptor expression in diabetic rats compared with control rats. This might be due to the duration and severity of the disease, as recently observed in a rat model of diabetic nephropathy.[4] Moreover, an increase in transforming growth factor-alpha (TGF-α), as observed in other diabetic models, might contribute to VEGF upregulation without affecting VEGF receptor expression.[16-18] Recently, an increased concentration of plasma VEGF, but not flt-1, was observed in type 2 diabetic patients with no complications, and diabetes-related atherosclerotic vascular disease was associated with a further increase in plasma VEGF and with an overexpression of flt-1.[8] The lack of flt-1 and flk-1 mRNA overexpression in diabetic hearts might be one of the mechanisms for this possible resistance to VEGF.

The discrepancy between our findings and those observed in short-term diabetic rats might be due to the different duration of diabetes. In fact, the reduction of VEGF in a short-term experimental model of diabetes[9] was explained by the authors as a consequence not only of the deficit of the insulin-induced VEGF production, but also of the still low hypoxic stimulation that generally characterized the first phases of STZ-induced diabetes in rats.[2] In our 90-day experimental diabetes, both the longer hyperglycemia through glycosylation end products or oxygen free radicals and mainly the greater hypoxia can produce the increase of VEGF levels in the heart. The delay in VEGF overproduction, together with no increase in its specific receptors, could explain the lack of collateral vessel formation in the typical diabetic heart.

Further investigations on VEGF involvement in coronary artery disease, from gene transcription to glycoprotein synthesis, are necessary to evaluate its role in all the phases of diabetic heart disease.

REFERENCES

1. Leung DW, Cachianes G, Kuang WJ, et al: Vascular endothelial growth factor is a secreted angiogenic mitogen. Science 246:1306-1309, 1989

2. Shweiki D, Itin A, Soffer D, et al: Vascular endothelial growth factor induced by hypoxia may mediate hypoxia-initiated angiogenesis. Nature 359:843-845, 1992

3. Neufeld G, Cohen T, Gengrinovitch S, et al: Vascular endothelial growth factor and its receptors. FASEB J 13:9-22, 1999

4. Cooper ME, Vranes D, Youssef S, et al: Increased renal expression of vascular endothelial growth factor and its receptor VEGFR-2 in experimental diabetes. Diabetes 48:2229-2239, 1999

5. Matsunaga T, Warltier DC, Weihrauch DW, et al: Ischemia-

induced coronary collateral growth is dependent on vascular endothelial growth factor and nitric oxide. Circulation 102:3098-3103, 2000

6. Shinohara K, Shinohara T, Mochizuki N, et al: Expression of vascular endothelial growth factor in human myocardial infarction. Heart Vessels 11:113-122, 1996

7. Waltenberger J, Lange J, Kranz A: Vascular endothelial growth factor-A-induced chemotaxis of monocytes is attenuated in patients with diabetes mellitus. Circulation 102:185-190, 2000

8. Belgore FM, Lip GYH, Lip PL, et al: Elevated levels of vascular endothelial growth factor (VEGF) and its receptor (sFlt-1) in diabetes. Eur Health J 21:P3487, 2000 (suppl, abstr)

9. Chou E, Suzuma I, Way KJ, et al: Decreased cardiac expression of vascular endothelial growth factor and its receptors in insulin-resistant and diabetic states. Circulation 105:373-379, 2002

10. Chomczynski P, Sacchi N: Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. Anal Biochem 162:156-159, 1987

11. Nahser PJJ, Brown RE, Oskarsson H, et al: Maximal coronary flow reserve and metabolic coronary vasodilatation in patients with diabetes mellitus. Circulation 91:635-640, 1995

12. Aronson D, Rayfield EJ, Chesebro JH: Mechanisms determining course and outcome of diabetic patients who have had acute myocardial infarction. Ann Intern Med 126:296-306, 1997

13. Waltenberger J, Lange J, Frank H, et al: The cellular response to VEGF-a is attenuated in diabetes mellitus in vivo and hyperglycemia in vitro. Circulation 102:882, 2000 (suppl S, abstr)

14. Natarajan R, Bai W, Lanting L, et al: Effects of high glucose on vascular endothelial growth factor expression in vascular smooth muscle cells. Am J Physiol 273:H2224-H2231, 1997

15. Losordo DW, Vale PR, Symes JF, et al: Gene therapy for myocardial injection of phVEGF165 as sole therapy for myocardial ischemia. Circulation 98:2800-2804, 1998

16. Gilbert RE, Wilkinson-Berka JL, Johnson DW, et al: Renal expression of transforming growth factor-β inducible gene-h3 (β-ig-h3) in normal and diabetic rats. Kidney Int 54:1052-1062, 1998

17. Mandriota SJ, Menoud PA, Pepper MS: Transforming growth factor β-1 downregulates vascular endothelial growth factor receptor 2/flk-1 expression in vascular endothelial cells. J Biol Chem 271: 11500-11505, 1996

18. Pertovaara L, Kaipainen A, Mustonen T, et al: Vascular endothelial growth factor is indiced in response to transforming growth factor-β in fibroblast and epithelial cells. J Biol Chem 269:6271-6274, 1994