Effect of simvastatin on expression of VEGF and TGF-β1 in atherosclerotic animal model of type 2 diabetes mellitus

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Abstract. Expression of vascular endothelial growth factor (VEGF) and transforming growth factor-β1 (TGF-β1) in atherosclerosis animal model of type 2 diabetes mellitus treated with simvastatin was investigated. Clean grade mature Sprague Dawley (SD) rats were divided into three groups: Normal control (n=10), model (n=13) and treatment group (n=13); low-dose simvastatin was administered. The changes of VEGF and TGF-β1 levels were analyzed by tail vein blood sampling. The relationship between levels of VEGF, TGF-β1 and treatment time was analyzed. The expression level of VEGF in the treatment group after 4 and 8 weeks of intervention was lower compared with the model group (P<0.05). The expression level of TGF-β1 in the treatment group after 8 weeks of intervention was higher than that in the model group (P<0.05). The expression level of VEGF in the treatment group after 8 weeks of intervention was lower than that after 1 week of intervention (P<0.05). The expression level of TGF-β1 was increased in the model group after 8 weeks of intervention compared with 1 week before and after the intervention (P<0.05). The expression level of TGF-β1 in the treatment group at 2, 4 and 8 weeks after intervention were significantly higher than that before intervention (P<0.05). The expression of TGF-β1 increased after 4 and 8 weeks after intervention compared with 1 week after intervention (P<0.05). The expression of VEGF was negatively correlated with TGF-β1 expression in the treatment group; negative correlation was found between VEGF and treatment time. There was a positive correlation between TGF-β1 and treatment time. VEGF and TGF-β1 may be involved in the development of type 2 diabetes (T2MD) atherosclerosis (AS). Simvastatin may play a therapeutic role in T2MD AS by downregulating VEGF and upregulating the expression of TGF-β1.

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Introduction

The incidence of type 2 diabetes (T2MD) is increasing as people's living standards increase, population ageing increases, and life structure changes. It is expected that T2MD will become the seventh largest cause of death in the world by 2030 (1,2). The major cause of death in patients with T2MD is major vascular complications (3). According to reports from the National Institutes of Health, 80% of patients with T2MD died of macrovascular complications directly (4). Therefore, how to treat T2MD macrovascular complications has become a global public health problem.

Atherosclerosis (AS) is the basis of T2MD macrovascular complications. It is prone to occur early in patients with T2MD, and the progression of AS is rapid and the prognosis of patients is poor. At present, the pathogenesis of AS in T2MD patients is not yet fully determined (5,6). Many researchers have found that the damage and dysfunction of the vascular endothelium, long-term chronic inflammatory response mediated by inflammatory factors and other reasons are important mechanisms for the occurrence and development of T2MD AS. Among them, vascular endothelial growth factor (VEGF) and transforming growth factor-β1 (TGF-β1) are the hot topics discussed in recent years. VEGF and TGF-β1 are abnormally expressed in patients with T2MD AS (5,7). Simvastatin is a competitive inhibitor of HMG-CoA reductase, and can block cholesterol synthesis (8). Studies have reported that simvastatin, in addition to regulating blood lipids, also has the effects of inhibiting proliferation of vascular smooth muscle cells, stabilizing AS plaques, resisting oxidative stress, preventing platelet aggregation, and improving endothelial function (9,10). However, its specific mechanism of action has not yet been studied clearly.

In this study, a rat model of T2MD was established to observe the effect of simvastatin on the expression of VEGF and TGF-β1 in rats and to investigate the mechanism of simvastatin.

Materials and methods

Research objects. A total of 40 clean grade mature Sprague Dawley (SD) rats were purchased from Animal Center of Xinjiang Medical University [production license SCXK (new) 2003-0001] and were fed with normal dry feed (Beijing Zhecheng Technology Co., Ltd., Beijing, China). SD rats were

~3 weeks old with an average age of 21.3±1.5 days, weight 150-160 g, average body weight 154.1±12.2 g, rearing temperature was 20-27˚C, relative humidity 70±10%, separate feeding in the terrarium, changing the litter every morning and evening, environmental noise below 85 dB, ammonia concentration not exceeding 20 ppm, ventilation 8-12 times per hour, fluorescent lamp light every 12 h, free food intake, free drinking water, feeding box was replaced 1-2 times a week. The humidity in the feeding box did not exceed 10%. The water bottle was changed 1-2 times a week. Ten random-number table methods were used to select 10 rats as normal control groups, and the remaining 30 were used to establish T2MDAS rat models. After successful modeling, they were randomly divided into model and treatment group. The study was approved by the Ethics Committee of Liaocheng People's Hospital (Liaocheng, China).

Rat model establishment. Thirty rats were used to establish T2MD AS rat model: High-fat diet (Keaoxieli Co., Ltd., Beijing, China) for 8 weeks, immediately after 8 weeks of feeding, fasted for 12 h, and given a single intraperitoneal injection of Streptozocin solution (Shanghai Baoman Biotechnology Co., Ltd., Shanghai, China) 45 mg/kg, after 72 h measured tail vein blood glucose, fasting blood glucose ≥7.0 mmol/l, and postprandial blood glucose ≥11.1 mmol/l was set as the evaluation criteria for successful T2MD model. Establishment of AS rat model: T2MD successfully modeled rats were intragastrically administered with vitamin D3 injection (SFDA approval no. 31021259; Wuhan Dongkangyuan Technology Co., Ltd., Wuhan, China), and were administered at a total dose of 500,000 U/kg for 3 days. The normal control group was fed with normal dry feed.

Treatment method. After simvastatin tablets (Shenzhen Roark Standards Technology Co., Ltd., SFDA Approval no. H20123114) was ground into a powder, 0.9% physiological saline was formulated into a 1 mg/ml suspension, and the treatment group was given a 10 mg/kg/day simvastatin suspension intragastrically for drug intervention. The normal control and model group were given 0.9% physiological saline 5 mg/kg/day gavage as a control. Treatment was performed for 8 weeks. The normal control group was fed with normal dry feed, and the model and treatment group were still fed with high-fat diet.

ELISA. Serum VEGF and TGF-β1 were detected by ELISA. The procedure was with reference to the instructions of the kit. VEGF and TGF-β1 kits were purchased from Shanghai Jing Kang Biological Engineering Co., Ltd. (Shanghai, China).

Observation indicators. The changes of VEGF and TGF-β1 levels before and after modeling (before intervention), 1, 2, 4, and 8 weeks after intervention were analyzed by tail vein blood sampling. The relationship between VEGF, TGF-β1 levels and treatment time was analyzed.

Statistical analysis. SPSS 19.0 (SPSS, Inc., Chicago, IL, USA) was adopted. Enumeration data are expressed as rate, and rates were compared by using the χ^2 test. The measurement data are expressed as mean ± SD. Analysis of variance was used for comparison among multiple groups, LSD tests were used for pairwise comparison, and repeated-variance measurement experiments or paired t-tests were used as comparison at different times within the group. The correlation between VEGF and TGF-β1 levels was analyzed by using Pearson's correlation. $P<0.05$ was statistically significant.

Results

Modeling results. T2MD rat models were first constructed in 30 rats. A total of 28 rats were successfully modeled. The success rate of modeling was 93.33%. Then AS rat model was established. Finally, 26 rats were successfully modeled. The success rate of modeling was 92.86%. There was no difference in body weight, fasting blood glucose, VEGF and TGF-β1 between the three groups before modeling ($P>0.05$). After successful modeling/before intervention, fasting blood glucose, VEGF, and TGF-β1 in the normal group were not different from those before modeling. Body weight, fasting blood glucose, VEGF, and TGF-β1 were increased in the model group and the treatment group at different degrees ($P<0.05$). The weight of rats before intervention in the normal group was higher than that before modeling ($P<0.05$). The vital signs before and after modeling in the three groups of rats are shown in Table I.

Changes of serum VEGF levels in three groups before and after intervention. There was no difference in serum VEGF levels between the three groups before modeling ($P>0.05$). Analysis of variance showed there were differences in the expression of VEGF among three groups of rats before intervention, 1, 2, 4, and 8 weeks after intervention ($P<0.05$). The LSD test results showed that the VEGF expression levels in the model and the treatment group at the five time-points were higher than those in the normal control group ($P<0.05$). The expression level of VEGF in the treatment group was lower than the model group after 4 weeks and 8 weeks after the intervention ($P<0.05$), and there was no difference at other time-points ($P>0.05$). The results of repeated analysis of variance at different time-points within the group showed that the expression levels of VEGF in the normal control group and model group had no significant changes before and after the intervention ($P>0.05$). The expression level of VEGF in the treatment group after 4 and 8 weeks of intervention was lower than that before the intervention ($P<0.05$). The expression level of VEGF in the treatment group after 8 weeks of intervention was lower than that after 1 week ($P<0.05$), and there was no difference between any of the other time-points ($P>0.05$) (Table II and Fig. 1).

Changes of serum TGF-β1 levels in three groups before and after intervention. There was no difference in the levels of serum TGF-β1 between the three groups before modeling ($P>0.05$). The analysis of variance showed that the expression levels of TGF-β1 of the three groups of rats were different before the intervention, 1, 2, 4, and 8 weeks after the intervention ($P<0.05$). The results of LSD showed that the expression levels of TGF-β1 in the model and the treatment group at the five time-points were higher than those in the normal control group ($P<0.05$). The expression level of TGF-β1 in the treatment was higher than that in the model group after

Table I. Vital signs before and after modeling in three groups of rats.

Items	Groups			Statistics	P-value
	Normal control	Model	Treatment		
Quantity	10	13	13		
Sex				0.020	0.981
Male	5	7	7		
Female	5	6	6		
Weight before modeling (g)	153.7±19.9	155.5±12.1	153.1±12.4	0.092	0.912
Weight before intervention (g)	261.8±34.5	385.3±56.4[a]	379.5±54.3[a]	23.66	<0.001
Fasting blood glucose before modeling (mmol/l)	4.26±1.02	4.28±1.01	4.24±1.03	0.005	0.995
Fasting blood glucose before intervention (mmol/l)	4.18±0.68	12.63±4.32[a]	12.56±4.27[a]	18.88	<0.001
Pre-modeling VEGF (μg/l)	76.03±12.14	77.12±11.49	76.59±12.34	0.023	0.977
	77.47±11.69	329.14±68.47[a]	332.29±67.56[a]	68.09	<0.001
Pre-intervention VEGF (μg/l)	3.27±0.79	3.29±0.78	3.31±0.80	0.007	0.993
	3.38±0.87	9.22±2.13[a]	9.26±2.12[a]	35.53	<0.001

[a]Comparison within group, P<0.05 compared with before modeling. VEGF, vascular endothelial growth factor.

Table II. Changes of serum VEGF levels in rats before and after intervention (μg/l).

Items	Groups			Statistics	P-value
	Normal control	Model	Treatment		
Quantity	10	13	13		
Before intervention	77.47±11.69	329.14±68.47[c]	332.29±67.56[c]	136.10	<0.001
1 week after intervention	77.06±11.32	332.44±67.58[c]	311.33±58.42[c]	74.18	<0.001
2 weeks after intervention	74.27±11.48	337.27±67.69[c]	296.69±51.04[c]	82.31	<0.001
4 weeks after intervention	73.29±11.56	344.55±67.98[c]	283.27±44.53[a,c,d]	90.76	<0.001
8 weeks after intervention	75.33±11.23	350.59±67.84[c]	254.28±45.16[a-d]	88.29	<0.001

[a]P<0.05 before intervention in the same group; [b]P<0.05 after 1 week of intervention in the same group; [c]compared with the control group at the same time-point (P<0.05); [d]P<0.05 compared with the model group at the same time-point. VEGF, vascular endothelial growth factor.

8 weeks of intervention (P<0.05), and there was no difference at other time-points (P>0.05). The results of repeated analysis of variance at different time-points within the group showed that the expression level of TGF-β1 in normal control rats did not change significantly before and after intervention (P>0.05). The expression level of TGF-β1 in the model group 8 weeks after intervention was higher than that before intervention and 1 week after intervention (P<0.05), and there was no difference in the expression level of TGF-β1 at other time-points in the model group (P>0.05). The expression level of TGF-β1 in the treatment group after 2, 4 and 8 weeks after intervention was higher than before intervention (P<0.05). After 4 and 8 weeks of intervention, the expression level of TGF-β1 was higher than that after 1 week of intervention (P<0.05). There was no difference between the other time-points in the treatment group (P>0.05) (Table III and Fig. 2).

Correlation analysis of VEGF and TGF-β1. Pearson's correlation analysis showed that the expression of VEGF was negatively correlated with TGF-β1 expression in the treatment group (r=-0.712, P=0.015). Negative correlation was found between VEGF and treatment time (r=-0.769, P=0.003). There was a positive correlation between TGF-β1 and treatment time (r=0.812, P=0.001). (Data not shown).

Discussion

Studies have reported that simvastatin can mediate CD40-CD40L signaling pathway in diabetic rats, reduce inflammation in arterial walls of diabetic rats, and improve vascular endothelium and its function in rats (11). However, there are many factors that cause T2MD AS, in addition to CD40-CD40L-related factors, whether VEGF and TGF-β1 play an important role in the treatment of diabetic rats AS with simvastatin is not yet clear and was the focus of this study.

A T2MD AS rat model was established in 2 steps. Clean SD rats were used to establish a T2MD rat model by feeding of high-fat diet for 8 weeks and using Streptozocin solution

Table III. Changes of serum TGF-β1 levels in rats before and after intervention (μg/l).

Items	Groups			Statistics	P-value
	Normal control	Model	Treatment		
Quantity	10	13	13		
Before intervention	3.38±0.87	9.22±2.13[c]	9.26±2.12[c]	35.53	<0.001
1 week after intervention	3.42±0.85	9.37±2.22[c]	9.97±2.22[c]	37.61	<0.001
2 weeks after intervention	3.40±0.86	9.85±2.21[c]	11.25±2.38[a,c]	47.30	<0.001
4 weeks after intervention	3.45±0.81	10.34±2.16[c]	12.01±2.44[a-c]	55.58	<0.001
8 weeks after intervention	3.58±0.80	11.20±2.14[a-c]	14.28±3.46[a-d]	46.00	<0.001

[a]P<0.05 before intervention in the same group; [b]P<0.05 after 1 week of intervention in the same group; [c]compared with the control group at the same time-point (P<0.05); [d]P<0.05 compared with the model group at the same time-point. TGF-β1, transforming growth factor-β1.

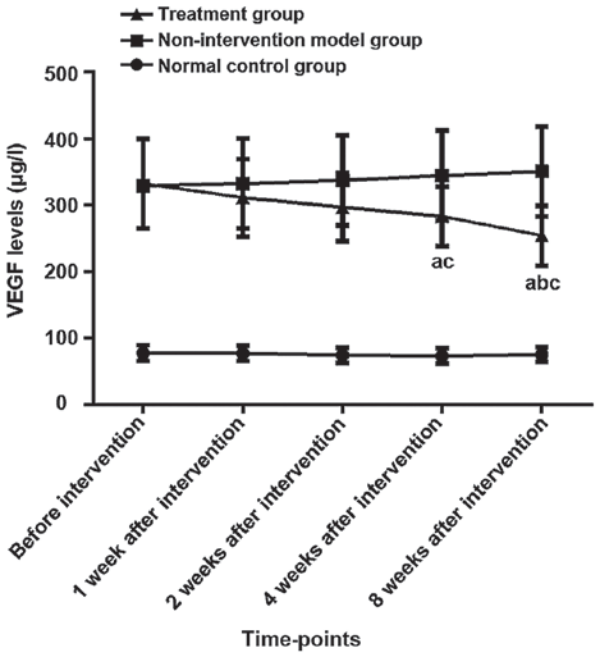

Figure 1. Changes of serum VEGF levels in three groups of rats before and after intervention. The expression of VEGF in the three groups before the intervention, 1, 2, 4 and 8 weeks after the intervention were all different (P<0.05). The expression level of VEGF in the non-intervention model and the treatment group at the five time-points was higher than that in the normal control group (P<0.05). The expression level of VEGF was lower in the treatment group than in the non-intervention model group 4 and 8 weeks after intervention (P<0.05). The expression of VEGF in the treatment group 4 and 8 weeks after intervention was lower than that before the intervention (P<0.05). The expression level of VEGF in the treatment group 8 weeks after intervention was lower than that of 1 week after intervention (P<0.05). [a]P<0.05 compared with pre-intervention; [b]P<0.05 compared with 1 week after intervention; [c]P<0.05 compared with no intervention model group at the same time-point. VEGF, vascular endothelial growth factor.

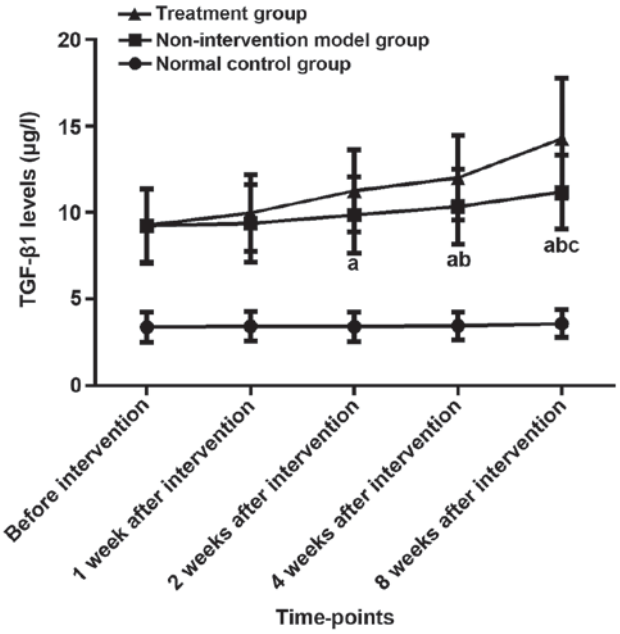

Figure 2. Changes of serum TGF-β1 levels in the three groups before and after intervention. The expression of TGF-β1 in the three groups before the intervention, 1, 2, 4 and 8 weeks after the intervention were all different (P<0.05). The expression levels of TGF-β1 in the non-intervention model and the treatment group at the five time-points were higher than those in the normal control group (P<0.05). The expression level of TGF-β1 in the treatment group was higher 8 weeks after the intervention than in the non-intervention model group (P<0.05). The expression level of TGF-β1 in the non-intervention model group was higher than that before the intervention and 1 week after the intervention (P<0.05). There was no significant difference in the expression level of TGF-β1 at the other time-points in the non-intervention model group (P>0.05). The expression level of TGF-β1 in the treatment group was higher at 2, 4, and 8 weeks after intervention than that before the intervention (P<0.05), and the expression level of TGF-β1 after 4 and 8 weeks after intervention was higher than that at 1 week after intervention (P<0.05). [a]P<0.05 compared with pre-intervention; [b]indicates P<0.05 compared with 1 week after intervention; [c]P<0.05 compared with no intervention model group at the same time-point. VEGF, vascular endothelial growth factor; TGF-β1, transforming growth factor-β1.

to destroy rat islet B cells. High-dose vitamin D disrupted the integrity of rat artery wall and replicated AS pathological model. After successful modeling, body weight and fasting blood glucose were significantly increased in rats, and VEGF and TGF-β1 were also significantly elevated. Therefore, we initially speculated that VEGF and TGF-β1 is involved in the development of rat T2MD AS. Then we used low-dose simvastatin to treat 13 T2MD AS rats in the treatment group. With the increase of treatment time, VEGF showed a decreasing trend. Until 4 weeks after treatment, the VEGF levels in the treatment group rats showed a significant difference compared with those before

the intervention, and there was also a statistically significant difference in VEGF levels between the treatment and the model group, indicating that simvastatin can improve VEGF levels in T2MD rats. The occurrence of AS in humans or rats causes insufficient blood supply to the arteries, resulting in endothelial cells secreting higher VEGF (8,12). Some studies have reported (13) that VEGF can activate the VEGFR2/KDR/flk1 signaling pathway, increase vascular permeability, promote adhesion of inflammatory cells in the blood vessel wall, and infiltration of inflammatory cells, promote neovascularization, and aggravate AS conditions. VEGF plays an important role in the occurrence of T2MD AS. In the present study, the results of TGF-β1 detection found that with the increase of treatment time, TGF-β1 showed an upward trend. After 2 weeks of treatment, serum TGF-β1 levels were statistically different from those before the intervention, indicating that simvastatin can increase TGF-β1 levels. Correlation analysis showed that VEGF was negatively correlated with treatment time, and TGF-β1 was positively correlated with treatment time. The inflammatory response is an important cause of plaque instability in AS, so improving the excessive inflammatory response is an important process for the treatment of AS (14,15). TGF-β1 has a strong non-specific anti-inflammatory effect (16). It was reported (17) that TGF-β1 can control the local inflammatory response due to AS by downregulating E-selectin and inhibiting the activity of $CD4^+$ T cells. The proliferation and migration of endothelial cells and the activation of the coagulation system due to endothelial cell damage are also important causes of AS (18,19). Some reports (20) indicated that TGF-β1 can activate the TGF-β1/ALK5/Smad3 signaling pathway and promote the expression of endothelin-1, thus hindering the proliferation and migration of endothelial cells. It has also been reported (21) that TGF-β1 can promote endothelial cells to express more prostacyclin, inhibit platelet aggregation, and prevent thrombosis. Therefore, TGF-β1 also plays an important role in the development of T2MD AS. Simvastatin can improve AS by improving the levels of VEGF and TGF-β1 in serum of T2MD rats. However, the trial of simvastatin in the treatment of diabetes generally lasted for more than 2 years. Since the animal model was used as the subject for analysis in this experiment, the analysis results only represented short-term therapeutic effects, and the long-term treatment effect could not be evaluated, and whether it has the same effect in human still needs to be studied.

In conclusion, VEGF, TGF-β1 may be involved in the occurrence of T2MD AS, and simvastatin may play a therapeutic role in T2MD AS by downregulating VEGF and upregulating TGF-β1 expression.

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Availability of data and materials

The datasets used and/or analyzed during the present study are available from the corresponding author on reasonable request.

Authors' contributions

HW wrote the manuscript. HW, JL and XF constructed T2MD rat models. YL and QX were responsible for ELISA. LS helped with statistical analysis. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The study was approved by the Ethics Committee of Liaocheng People's Hospital (Liaocheng, China).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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