·Clinical Research·

Elevation of serum apelin-13 associated with proliferative diabetic retinopathy in type 2 diabetic patients

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Abstract

• AIM: To compare apelin-13, a ligand of G-protein-coupled receptor which has been shown to be involved in retinal angiogenesis, and vascular endothelial growth factor (VEGF) serum levels in type 2 diabetes mellitus (T2DM) with or without retinopathy, and to investigate the relationship between the serum concentration of apelin-13 and diabetes retinopathy.

• METHODS: Sixty-nine patients with T2DM were enrolled. Of the 69 patients, 16 had proliferative diabetic retinopathy (PDR group), 23 had non-PDR (NPDR group) and 30 had no retinopathy (T2DM group). Subjects´ information, including demographics, medical history, and use of medications were recorded. Their serum samples were collected for measuring the levels of C-reactive protein (CRP), serum lipid and glycosylated hemoglobin. Apelin-13 and VEGF serum levels were measured by enzyme-linked immunosorbent assay. Kruskal-Wallis test and one-way ANOVA were used to compare the differences among these groups. Chi-square test was used to assess categorical variables. Correlations between variables were investigated by Spearman rho correlation test and stepwise regression analysis. All statistical analyses were performed through SPSS 17.0 software.

• RESULTS: Sex, age, body mass index (BMI), blood pressure, CRP, hemoglobin A1c (HbA1c) have no significantly difference in the three groups. Serum level of apelin-13 was significantly elevated in PDR group as compared with T2DM group (P=0.041). Differences of VEGF serum concentration in the three groups were statistically significant (P=0.007, P=0.007 and P<0.001, respectively). Spearman rho correlation test showed that serum apelin-13 was positively correlated with BMI, serum triglycerides, VEGF, but not with age, duration of diabetes, blood pressure, CRP, HbA1c and total-cholesterol. Stepwise regression analysis showed that BMI also significantly associated with serum apelin-13 (P=0.002), while VEGF and serum triglycerides were irrelevant.

• CONCLUSION: This study elucidated a positive association of apelin-13 serum level with PDR, but not with VEGF. Apelin-13 may influence the promotion of PDR but unrelated with VEGF.

• KEYWORDS: diabetic retinopathy; apelin-13; vascular endothelial growth factor
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INTRODUCTION

Diabetic retinopathy (DR) is a serious microvascular complication of diabetes mellitus (DM) and is one of the leading causes of blindness in working-age population in the industrialized world [1,2]. Retinal neovascularization (RNV) is the most important clinical features of proliferative DR (PDR). A number of angiogenic factors including cytokines, inflammatory cells, growth factors, *etc*. have been identified to play important roles in the pathogenesis of RNV [3-6]. Vascular endothelial growth factor (VEGF) is considered to be a key molecule during pathogenesis of

RNV. Serum levels of VEGF are detected to be associated with the development of DR [7]. Although anti-VEGF therapies have proven to be effective in treating DR [8,9], the treatments show a transient efficacy[10]. Besides, VEGF levels were not entirely relevant to the severity of DR [4]. Thus it suggested that there would be some other substances which also play roles in the promotion of DR rather than upregulating VEGF expression[11].

Apelin is a number of adipokines and has gained a lot of attention after the first notion that it was involved in metabolic disorders[12]. Apelin was produced and secreted by both human and mouse white adipose tissue [13]. Studies have shown that apelin was involved in vascular pathophysiology[14] and act as an angiogenic factor stimulating retinal endothelial cells' proliferation, migration and vascular tube formation[15,16]. Apelin mRNA was highly expressed in vascular system, particularly in vascular endothelial cells [17]. Recent studies discovered a possible involvement of apelin signaling in retinal angiogenesis [16,18-20]. Furthermore, studies found that inhibition of apelin/apelin receptor (APJ) system prevented rapid abnormal vessel growth, deficiency of apelin successfully inhibited hypoxia-induced retinal angiogenesis in mice despite upregulation of VEGF. Apelin small and interfering RNA suppressed endothelial cells proliferation independent of VEGF/VEGF receptor 2 signaling pathway[21]. Animal studies demonstrated that inhibition of apelin-APJ system facilitated retinal vessel maturation in ischemic retinopathy model, and inhibition of apelin expression switched endothelial cells from proliferative to mature state in pathological retinal angiogenesis[22]. Treatment with apelin specific antagonist F13A produced a marked reduction of VEGF mRNA expression in the retina of diabetic rats which were induced by intraperitoneal injections of streptozotocin (STZ: 60 mg/kg body weight)[23]. Clinical study found that apelin concentration in vitreous were significantly higher in patients with PDR than nondiabetic patients [24]. Thus, apelin may be involved in development of PDR. However, evidences on the relation between serum concentration of apelin and DR are still unclear, and more consistent data are warranted.

Apelin exists several subtypes, such as apelin-36, apelin-17, apelin-13 and post-translation of modified apelin-13 and apelin-12. All bioactivity is thought to reside in the terminal 13 aa fragment (apelin-13)[15,23,25]. So, we conducted our study to examine the association between apelin-13 serum levels and DR.

SUBJECTS AND METHODS

Patients Inclusion and Exclusion This study was approved by Ethics Committee of Xi'an Ninth Hospital Affiliated to Medical College of Xi'an Jiaotong University, all of the procedures were performed in accordance with ethical approval institutional guidelines. Before the study, informed consent for all examinations and procedures were obtained from each subject.

All patients were recruited from Department of Ophthalmology and Endocrinology during May 2012 to June 2013. According to 2006 World Health Organization standard of type 2 DM (T2DM), all subjects were examined and diagnosed whether or not suffering diabetic complications, including DR, diabetic nephropathy, diabetic neuropathy and diabetic angiopathy. Patients with the following conditions were excluded from this study: type 1 diabetes, T2DM complicated with infection and other systemic diseases that may affect the test results, such as systemic lupus erythematosus, rheumatic arthritis, diabetic nephropathy, presence of any psychological or neurological disorder.

Ocular examination including slit-lamp biomicroscopy and ophthalmoscopy was performed on patients. Subjects with the following ocular disease were excluded: glaucoma, uveitis, pigmentary degeneration, wet age-related macular degeneration and dense cataract which prevented an ophthalmoscopic examination of the ocular fundus. If retinal hemorrhage, exudation and microaneurysm were occured in patients, fundus fluorescein angiography and fundus color photography (fundus camera TRC-50EX; Topcon, Tokyo, Japan) were conducted further. The severity of DR was assessed and patients were divided into non-PDR (NPDR) group and PDR group according to international classification for DR. Those patients without DR were enrolled in T2DM group. Cases were selected in 3 groups based on age and sex. All patients were subjected to thorough clinical examination and laboratory investigations to establish their diagnosis and to exclude other associated pathological conditions.

Study Protocol

Patients information and general examination Age, gender, body height, body mass, duration of diabetes, diseases history (include ocular diseases and system diseases) and use of medications of all patients were recorded. Body mass index (BMI) was calculated. Patients' systolic blood pressure (SBP) and diastolic blood pressure (DBP) were measured in sitting position by mercury sphygmomanometer, and later the patients had rested for at least 15min in the morning on the blood collected day.

Apelin-13, vascular endothelial growth factor and other factors tests in peripheral blood Eight hours after eating, 6 mL blood samples from subjects was collected in sterile dry tubes. 3 mL blood was immediately centrifuged for 10min at 4℃ at 3500 rpm for serum collection. The serum was collected in 2 copies and then stored at -80℃ until further analysis. Apelin-13 and VEGF were analyzed in biochemistry laboratory at the Center for Disease Control and prevention of Shaanxi Province when all samples were

Table 1 Composition of the study population

Parameters	T2DM (n=30)	NPDR (n=23)	PDR (n=16)	P
Age (a)	57.2±4.7	56.9±3.0	55.3±4.7	0.248
Sex				
F	12 (40%)	9 (40%)	7 (40%)	>0.99
M	18 (60%)	14 (60%)	9 (60%)	
Duration of diabetes (a)	6.2±3.5	10.3±4.6[a]	10.8±4.2[a]	<0.001
BMI (kg/m^2)	22.92±1.82	24.08±2.15	23.41±2.37	0.138
SBP	134.57±9.59	136.30±10.89	135.13±8.16	0.715
DBP	81.93±5.60	84.00±7.67	82.78±7.15	0.586
HbA1c	8.09±1.53	8.34±1.22	8.43±1.51	0.722
CRP	3.33±2.07	4.19±2.68	5.09±2.38	0.024
Total-cholesterol	4.67±0.83	5.23±0.77[a]	5.92±1.43[a,b]	0.002
Triglycerides	1.07±0.44	1.75±1.14[a]	2.23±0.84[a]	<0.001
Apelin (ng/mL)	3.70±1.49	3.69±1.25	4.55±1.07[a]	0.045
VEGF (pg/mL)	105.91±30.32	129.14±24.36[a]	156.46±36.24[a, b]	<0.001

BMI: Body mass index; SBP: Systolic blood pressure; DBP: Diastolic blood pressure; HbA1c: Haemoglobin A1c; CRP: C-reactive protein; VEGF: Vascular endothelial growth factor. [a]Significant *vs* T2DM group. [b]Significant *vs* NPDR group.

collected. The rest 3 mL blood samples collected and sent immediately to the hospital laboratory was used for HbA1c, lipid and CRP test.

All reagents and standards were prepared as instructed in the kit. Serum levels of apelin-13 and VEGF were determined with commercial human enzyme linked immunosorbent assay kits (human apelin-13 ELISA Kit; Uscnlife Science & Technology Company, Missouri, human VEGF ELISA Kit; RapidBio Lab, Calabasas, CA, USA) used according to the manufacturers' instructions. The results were calculated as directed in the kit. Each assay was performed in duplicate.

Statistical Analysis All statistical analyses were performed using the statistical package for social sciences 17.0 software package (SPSS for Windows, version 17.0; Chicago, IL, USA). Data were presented as mean±standard deviation (SD). Kruskal Wallis test and one-way ANOVA were used to make comparisons between pairs of groups. Chi-square test was used to assess categorical variables. Correlations between variables were investigated by Spearman rho correlation test. Significant variables from Spearman's correlation analyses will further analyzed by multiple regression. A two-tailed P values of <0.05 was accepted as statistically significant.

RESULTS

Our study totally enrolled 69 patients in three groups. The clinical characteristics of the three groups were presented in Table 1. No significant differences were found in sex, age, BMI, blood pressure, CRP and HbA1c among groups. The duration of diabetes in NPDR and PDR group was statistically significant longer than T2DM group (P<0.05). Serum levels of VEGF in PDR group were found higher compared to NPDR group and T2DM group (P=0.007, P< 0.001, respectively). VEGF levels in NPDR group were found higher than T2DM group(P=0.007). Apelin-13 serum level in PDR group were significantly higher than T2DM group (P=0.041). No significant differences of apelin-13 were found between NPDR group and T2DM group.

Table 2 Correlation between different variables and apelin used Spearman rho correlation coefficient

Parameters	Correlation coefficient	P
Age (a)	-0.129	0.290
Duration of diabetes	0.163	0.180
BMI (kg/m^2)	0.380[a]	0.001[a]
SBP	0.052	0.067
DBP	-0.021	0.866
HbA1c	-0.091	0.456
CRP	-0.007	0.956
Total-cholesterol	0.152	0.214
Triglycerides	0.267[a]	0.027[a]
VEGF (pg/mL)	0.253[a]	0.036[a]

BMI: Body mass index; SBP: Systolic blood pressure; DBP: Diastolic blood pressure; HbA1c: Haemoglobin A1c; CRP: C-reactive protein; VEGF: Vascular endothelial growth factor. [a]Significant changes (P<0.05).

Table 3 Multiple linear regression analysis. Serum apelin concentration is the dependent variable

Parameters	Standardized regression coefficients	t	P
Triglycerides	0.163	1.339	0.185
VEGF (pg/mL)	0.178	1.471	0.146
BMI (kg/m^2)	0.369	3.253	0.002[a]

VEGF: Vascular endothelial growth factor; BMI: Body mass index. [a]Significant changes (P<0.05).

Spearman's correlation analyses demonstrated that apelin-13 serum levels were associated with BMI, triglycerides and VEGF (Table 2). Multiple regression analysis with all the significant variables from the Spearman's correlation analyses confirmed that only BMI remained significantly associated with serum apelin-13 (P=0.002; Table 3).

DISCUSSION

The results of this study demonstrated that apelin-13 serum concentrations in PDR patients were significantly higher compared to T2DM individuals. Serum levels of apelin-13 were not associated with VEGF.

Apelin is a crucial factor for hypoxia-induced retinal angiogenesis [21,26,27]. Previous study found that apelin level were similar in NPDR, PDR and patients without DR. But these data showed that the level of apelin tended to be elevated in PDR group compared to T2DM and NPDR group (5.13±4.07 ng/mL, 4.45±3.68 ng/mL and 3.62±1.44 ng/mL, respectively)[28]. Furthermore, Tao *et al* [24] found that apelin concentrations in vitreous in PDR patients were significantly higher than nondiabetic patients, but plasma concentrations of apelin did not vary significantly between both groups. Apelin level differences between intraocular fluid and serum maybe caused by the distinct local production of apelin, as an autocrine function maybe exist in retinal vascular endothelial cells. These results were contradictory with our study. In this study, we found serum concentrations of apelin-13 was significantly elevated in PDR group than in T2DM group, while no statistically significant difference was found between PDR group and NPDR group. The inconsistency maybe due to different patients enrollment criteria.

PDR refers to a severe stage of DR and is characterized by vitreous hemorrhage, retinal hemorrhage and neovasculatization originating from the retina. Previous studies found that apelin signaling may be involved in retinal angiogenesis [16,18-20]. Apelin plays a role in stimulating retinal endothelial cells' proliferation, migration and vascular tube formation[15,16]. Otherwise, the underlying mechanisms which apelin was involved in retinal angiogenesis was unclear. Some studies found that apelin was related with oxidative and inflammation markers, and can be up-regulated by TNF-α which is a marker of low-grade inflammation [29-31]. It was reported that administration of APJ antagonist decreased inflammatory cytokines including TNF-α [32]. Thus we speculated that the apelin pathway may play a role in monitoring systemic inflammatory response. C-reactive protein (CRP) is produced by the liver that increases in the presence of inflammation or tissue damage, and is more sensitive than white blood cells or erythrocyte sedimentation rate. Compared with CRP, the detection of high sensitive CRP (hs-CRP) is more sensitive and accurate and has been widely applicated in clinic for monitoring inflammation. Previous studies suggested that DR may be a kind of low grade and subclinical inflammatory disease [3-6]. And inflammation is one of the promoting factors of retinal angiogenesis. Thus, we analyzed the relationship between CRP and apelin, but we failed to find their correlation in this study. Our result was different from another study which found apelin was correlated with hs-CRP [33]. This inconsistency may be due to the higher sensitivity of hs-CRP than CRP, or partly due to the application of some drugs which may affect the serum level of CRP.

Moreover, Lu *et al* [23] found that mRNA and protein levels of apelin, glial fibrillary acidic protein (GFAP) and VEGF were significantly increased in the retina of diabetic rats. Simultaneously, apelin induced GFAP and VEGF expression. Furthermore, apelin specific antagonist F13A suppressed both GFAP and VEGF expression *in vivo*. These results suggest that apelin may play a role in the progression of DR through up-regulation of VEGF and some other angiogenic factors *in vivo*. Moreover, Kasai *et al* [34] found that retinal vascularization and angiogenic responses to VEGF were remarkably decreased in apelin-knockout (apelin-KO) mice. The reduced responses to VEGF in apelin-KO mice were partially restored by apelin. Another research showed that apelin expression in retina was dramatically increased in oxygen-induced retinopathy model mice, and apelin deficiency hardly induced retinal angiogenesis despite the upregulation of VEGF *in vivo*[21]. These results indicate that apelin may participate in the regulation of VEGF-induced angiogenesis. A recent study found that apelin-13 enhanced migration, proliferation, and chemotaxis of glomerular endothelial cells. It also promoted permeability of glomerular endothelial cells and upregulated VEGF receptor 2 (VEGFR2) expression [35]. VEGFR2 can promote proliferation and chemotaxis, induce the permeability of endothelial cells through binding to VEGF. So, apelin may be attributed to angiogenesis promoting and permeability increasing *via* upregulation of VEGFR2 expression. Thus we speculated that high concentration of serum apelin-13 may correlate with serum VEGF. However, in our research we found that the apelin serum level was not correlate with VEGF. This was consistent with some previous studies [24,36,37]. The reason of this difference of relationship between apelin and VEGF was still unclear. Therefore, further studies are needed to investigate the relationship between apelin and VEGF.

Furthermore, apelin has a close relationship with metabolism. Previous studies found that apelin levels in serum were increased and associated with glucose homeostasis, and BMI in T2DM [29,38]. This was consistent with our result. Apelin was positively correlated with BMI, suggesting that apelin levels may associated with obesity. Previous study found that TNF-α mRNA and protein levels in the white adipose tissue was increased in obese patients, and can be used as an important autocrine and paracrine

adipose regulating factor [39]. It is reported that TNF-α up-regulated the level of apelin [31], thus DR was more prone to develop in obese patients. But other study found that obese patients with T2DM had significantly higher apelin levels than non-diabetic obese subject, confirming that increased apelin levels are directly associated with the presence of diabetes rather than obesity itself [38]. So, further research is needed. Furthermore, we fail to find an association among apelin and age, duration of the disease, blood pressure, HbA1c and lipid. This was inconsistent with other study [29]. This maybe partly due to the application of Antihypertensive drugs, hypoglycemic agents and lipid-lowering drugs.

The sample size in our study is small and limited. Further researches are needed to reveal the levels of apelin in vitreous humour in NPDR and PDR patients. In addition, the experiments whether a network of cooperation among other angiogenic factors with apelin exists are needed. Effect of apelin on proliferation, migration and capillary tube formation of human retinal vascular endothelial as well as the underlying mechanisms also need to be studied.

In conclusion, our data demonstrated that apelin-13 serum levels were elevated for patients with PDR. Apelin were significantly associated with BMI, but not with VEGF. These results indicated that elevated serum apelin levels maybe involved in the development of PDR. Apelin may offer a new therapeutic opportunity against RNV and still needs to be evaluated further.

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