A study on the expression of FGF-21 and NF-κB pathway in the tissues of atherosclerotic mice

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Abstract. – **OBJECTIVE:** To study the relationship between the expressions of fibroblast growth factor (FGF)-21 and NF-κB signal transduction pathway in the tissues of atherosclerotic mice.

MATERIALS AND METHODS: A total of 40 apoE-/- male mice at 8 weeks were selected and randomly divided into 4 groups. 10 mice in group A were normally fed with diet. 10 mice in group B were fed with high-fat diet. 10 mice in group C were fed with high-fat diet + pravastatin. 10 mice in group D were fed with high-fat diet + subcutaneous injection of exogenous recombinant FGF-21 protein. Another 10 C57BL/6J mice at 8 weeks were normally fed with diet (group E). They were killed after 12 weeks to collect retinal venous blood. ELISA method was applied to detect the levels of serum FGF-21, NF-κB, monocyte chemo attractant protein (MCP-1), matrix metalloproteinase (MMP)-9 and TNF-α. Immunohistochemical staining and RT-PCR method were applied to detect the expression of FGF-21 in aortic arch and liver tissues. RT-PCR method and Western blot method were applied to detect the expression of NF-κB, MCP-1, MMP-9 and TNF-α in aortic arch and liver tissues.

RESULTS: The levels of serum FGF-21, MCP-1, MMP-9 and TNF-α in group B were higher than those of group A and group E, and those of group C and group D were lower than those of group B (except FGF-21 in group D). The differences had statistical significance ($p<0.05$). The positive staining rates of FGF-21 in endothelial cells of aortic arch and liver tissues in group B were higher than those group A and group E, and those of group C and group D were lower than those of group B. The differences had statistical significance ($p<0.05$). The expression levels of FGF-21mRNA, NF-κB, MCP-1, MMP-9, TNF-αmRNA and protein in endothelial cells of aortic arch and liver tissues in group B were higher than those group A and group E, and those of group C and group D were lower than those of group B. The differences had statistical significance ($p<0.05$).

CONCLUSIONS: FGF-21 may participate in the occurrence of atherosclerosis (AS), which is related to the activation of NF-κB pathway. Lipid-lowering therapy can inhibit the activation of FGF-21 and NF-κB. Exogenous FGF-21 can also lower the activation of the NF-κB and interpose in atherosclerosis process.

Key Words:
     Atherosclerosis, Fibroblast growth factor-21, NF-ĐB.

Introduction

As the common pathological basis of diseases, such as cardiovascular and cerebrovascular diseases, metabolic syndrome, diabetes mellitus and hypertension[1], atherosclerosis (AS) involves many theories, including lipid infiltration, endothelial injury, medial smooth muscle cell proliferation and oxidative stress, and inflammatory reaction[2]. It is considered to be on the basis of heredity and the result of joint effects of multiple causes of disease and factors[3]. Fibroblast growth factor (FGF) has the function of endocrine factor, takes part in maintaining glucolipid metabolism balance of body, and regulates pathophysiological processes, such as cell proliferation, body development, vascular proliferation and wound healing[4]. FGF-21 is mainly expressed in liver and fat tissues. The expression level of circular FGF-21 in patien-

ts with coronary heart disease is obviously higher than that of normal population and it is closely related to disease severity and clinical prognosis[5]. The treatment of antiplatelet, lipid-lowering and anti-inflammation can reduce its expression level[6]. All of these indicate that FGF-21 may participate in the occurrence of AS. As the transcription factor with the effect of gene transcription regulation, NF-κB plays an important role in the process of inflammatory reaction, immune response, cell proliferation, transformation and apoptosis[7]. Various genes participating in the occurrence of AS are closely related to the activation state of NF-κB signal transduction pathway[8]. This study aimed to analyze the relationship between the expression of FGF-21 in the tissues of AS mice and NF-κB pathway.

Materials and Methods

Animal Materials and Experimental Groups

A total of 40 apoE^{-/-} healthy male mice at 8 weeks were selected, with the average body weight of (25±5) g. Purchased from Sangon Biotech Animal Experiment Center (Shanghai, China), they were fed normally and used to conduct experiment after adapting to environment for 1 week. They were randomly divided into 4 groups. 10 mice in group A were normally fed with diet. 10 mice in group B were fed with high-fat diet (88.5% normal mixed diet + 10% lard +1% cholesterol +0.5% bile salt from pig). 10 mice in group C were fed with high-fat diet + pravastatin (40 mg/kg/d). 10 mice in group D were fed with high-fat diet + subcutaneous injection of exogenous recombinant FGF-21 protein (100 μg/kg). Another 10 C57BL/6J mice at 8 weeks were normally fed with diet (group E) and they were killed after 12 weeks. Recombinant FGF-21 protein was the genetic engineering strain compounded by applying genetic engineering technology by Sigma-Aldrich Corporation (St. Louis, MO, USA) to construct the fusion expression of SUMO molecular chaperone and FGF-21, with the protein expression of more than 20%. It could react with FGF-21 monoclonal antibody, with the product purity reaching over 95% and recovery rate exceeding 60%. The study was approved by the Ethics Committee of Shanghai Chest Hospital. Signed written informed consents were obtained from all participants before the study.

Research Methods and Observation Indexes

After fasting overnight, 2500 g retinal venous blood was collected to centrifuge for 20 min. Supernatant was taken and stored at -80°C. ELISA method was applied to detect the levels of serum FGF-21, NF-κB, monocyte chemoattractant protein (MCP-1), matrix metalloproteinase (MMP)-9 and TNF-α. Kit was purchased from Jiangsu Beyotime Biotechnology Co., Ltd. (Haimen, Jiangsu, China) and ELISA was purchased from Bio-Rad Corporation (Hercules, CA, USA). They were used strictly according to the steps of the instructions. Immunohistochemical staining and reverse transcriptase-polymerase chain reaction (RT-PCR) method were applied to detect the expression of FGF-21 in aortic arch and liver tissues. RT-PCR method and Western blot method were applied to detect the expression of NF-κB, MCP-1, MMP-9 and TNF-α in aortic arch and liver tissues. Tissue slices were prepared with conventional method, with the thickness of 4 μm. Main steps of immunohistochemical staining: dimethyl benzene dewaxing, gradient ethanol hydration and antigen retrieval. 3% H_2O_2 solution was added to incubate at 27°C for 20 min. Normal goat serum working solution was added drop by drop and incubated at 27°C for 30 min. Rabbit anti-mouse FGF-21 monoclonal antibody first antibody (Beijing ZSGB-BIO Co., Ltd., Beijing, China) with the working concentration of 1:2000, was added drop by drop and then placed in wet box to incubate overnight at 4°C. Normal mouse IgG was taken to substitute first antibody as control. Goat anti-rabbit IgG polyclonal antibody second antibody (R&D Corporation, Minneapolis, MN, USA, with the working concentration of 1:500) was added drop by drop and then placed in wet box to incubate at 27°C for 20 min. Horseradish peroxidase-labeled streptavidin peroxidase working solution (Santa Cruz Corporation, Santa Cruz, CA, USA) was added drop by drop and then placed in wet box to incubate at 27°C for 20 min. Vibration and washing with phosphate buffered saline (PBS) were conducted for 5 min × 3 times. 3,3'diaminobenzidine tetrachloride (DAB) color developing, hematoxylin counterstaining, hydrochloric acid alcohol differentiation, ammonia water back to blue, dehydration of gradient ethanol, transparency with xylene, sealing sheet with neutral balsam, drying at room temperature, and observing under optical microscope. Result determination: semi quantitative method was applied according to staining intensity and the ratio of stained cells. When cytoplasm or cell nuclear yellow was dyed to be brown, it was considered to be positive, with 0 score

for no positive staining in staining intensity, 1 score for weak staining, 2 scores for moderate-intensity staining and 3 scores for strong staining. When the ratio of the number of positive cells was ≤5%, the score was 0, with 1 score for 6-25%, 2 scores for 26-50%, 3 scores for 51-75% and 4 scores for >75%. When the product of two items was 0-3 scores, it was considered to be negative. When the product of two items was 4-12 scores, it was considered to be positive. 5 samples were taken from aortic arch and liver tissues of each mouse, totaled 50 samples for each group. Main steps of RT-PCR method: total RNA of cells was extracted with conventional Trizol reagent to detect the concentration and purity with ultraviolet spectrophotometer. Reverse transcription kit was applied to compound cDNA. Shanghai Sangon Biotech Co., Ltd. (Shanghai, China) compounded primer sequences according to Gene Bank sequences, FGF-21: (F)5'-CTGGGG-GTCTACCAAGCATA-3',(R)5'-CACCCAG-GATTTGAATGACC-3'; NF-κB p65: (F)5'-A-AGATCAATGGCTACACAGG-3',(R)5'-C-CTCAATGTCTTCTTTCTGC-3'; MCP-1: (F)5'-ATGCAGGTCTCTGTCACG-3',(R)5'-CTA-GTTCTCTGTCATACTGGTCAC-3'; MMP-9: (F)5'-CACACAACGTCTTTCACTAC-3',(R)5'-CAA-GCTGATTGGTTCGAGTA-3';TNF-α: (F)5'-CGT-TGTAGCCAATGTCAAAGCC-3',(R)5'-TGCC-CAGATTCAGCAAAGTCCA-3';GAPDH(F): 5'- CGCGAGAAGATGACCCAGAT-3', R: 5'-GCACTGTGTTGGCGTACAGG-3'. Reaction system was 2 μl cDNA + 3 μl upper and lower primers, respectively + 0.5 μl Taq polymerase + 1 μl dNTPs + 3 μl MgCl$_2$ + 5 μl 10 × Buffer + 2.5 μl ddH$_2$O$_2$. Reaction conditions included 95°C for 5 min, 95°C for 30 s, 58°C for 30 s, 72°C for 60 s, a total of 30 cycles, and ending by 72°C for 10 min. 2% agarose gel electrophoresis was applied to identify PCR products. Gel imaging was applied to analyze ultraviolet spectroscopic imaging. Digital photographing was applied to conduct gray scale analysis and the result was expressed with $2^{-\Delta\Delta Ct}$. Main steps of Western blot method: after tissue homogenate, radioimmunoprecipitation assay (RIPA) lysis buffer was added, total cell protein was extracted, and rough quantification was conducted with Coomassie brilliant blue. Before detecting protein each time, β-actin antibody was used to conduct dose standardization detection for the protein of all samples. 30 μg total protein was taken and separated by 8% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) electrophoresis. The charged disengagement zone was transferred to polyvinylidene fluoride (PVDF) membrane and added with rabbit

anti-mouse NF-κB, monocyte chemoattractant protein-1 (MCP-1), matrix metallopeptidase-9 (MMP-9) and tumor necrosis factor- α (TNF-α) monoclonal antibody (1:2000, Sigma-Aldrich Corporation, St. Louis, MO, USA) to rest overnight. Goat anti-rabbit polyclonal antibody second antibody (1:500, Sigma Corporation, St. Louis, MO, USA) was added to incubate at room temperature for 4 h, followed by phosphate-buffered saline (PBS) washing and ECL color developing. Results were scanned for storage. Semi quantitative analysis was conducted with Lab Works 4.5 gel imaging software (Invitrogen, Carlsbad, CA, USA) and expressed with integral optical density (IOD).

Statistical Analysis

Statistical analysis was conducted by SPSS20.0 software (Version X; IBM, Armonk, NY, USA). Measurement data was showed by mean ± standard deviation. The inter-group comparison applied single-factor ANOVA analysis. Pair-wise comparison was tested by LSD-t method. Enumeration data was expressed with cases or (%). The inter-group comparison was tested with χ^2. $p<0.05$ indicated that the difference had statistical significance.

Results

Comparison of the Levels of Serum FGF-21, NF-κB, MCP-1, MMP-9 and TNF-α

The levels of serum FGF-21, NF-κB, MCP-1, MMP-9 and TNF-α in group B were higher than those of group A and group E, and those of group C and group D were lower than those of group B (except FGF-21 in group D). The differences had statistical significance ($p<0.05$), as shown in Table I.

Comparison of the Results of Immunohistochemical Staining

The positive staining rates of FGF-21 in endothelial cells of aortic arch and liver tissues in group B were higher than those group A and group E, and those of group C and group D were lower than those of group B. The differences had statistical significance ($p<0.05$), as shown in Table II.

Comparison of the Results of RT-PCR Method

The expression levels of FGF-21, NF-κB, MCP-1, MMP-9 and TNF-αmRNA in endothelial cells of aortic arch and liver tissues in group B were higher than those group A and group E, and

Table I. Comparison of the levels of serum FGF-21, NF-κB, MCP-1, MMP-9 and TNF-α (ng/ml).

Group	FGF-21	NF-κB	MCP-1	MMP-9	TNF-α
Group A	4.5±1.3	7.2±2.3	23.4±6.9	62.8±24.3	36.6±12.5
Group B	25.6±8.2	32.5±7.5	66.5±15.7	125.4±65.8	92.4±25.7
Group C	13.4±6.3	14.2±5.4	35.9±12.3	92.7±32.4	65.7±23.4
Group D	42.7±12.5	11.8±5.1	32.3±11.4	86.5±31.9	62.4±21.5
Group E	4.0±1.2	6.6±2.2	21.1±6.2	65.9±23.6	32.5±13.6
F	25.634	12.532	18.438	16.527	14.623
p	0.000	0.000	0.000	0.000	0.000

those of group C and group D were lower than those of group B. The differences had statistical significance ($p<0.05$), as shown in Table III.

Comparison of the Results of Western Blot Method

The expression levels of NF-κB, MCP-1, MMP-9 and TNF-α in endothelial cells of aortic arch and liver tissues in group B were higher than those group A and group E, and those of group C and group D were lower than those of group B. The differences had statistical significance ($p<0.05$), as shown in Table IV.

Discussion

The study[9] found that FGF-21 could promote the proliferation and migration of vascular smooth muscle cells during AS process. There is no heparin-binding domain in FGF-21 protein structure. After expression, it can secrete to enter into blood to play the function of regulating glucolipid metabolism[10]. Controlled by PPARαJ gene, FGF-21 can affect energy metabolism of body[11]. FGF-21 can activate ERK1/2 and Akt signal pathway, improve the functions of pancreatic acinar β cells, and increase insulin sensitivity[12]. In adipocytes, it can promote the expression of glu-

cosyl transferase 1 and increase the absorption of glucose[13]. FGF-21 can also inhibit obesity caused by high-fat diet, reduce the levels of total cholesterol and low-density lipoprotein, and increase the levels of apolipoprotein A1 and high-density lipoprotein[14]. This study concluded that the level of serum FGF-21 in group B was higher than that of group A and group E, and that of group C was lower than that of group B. Clinical studies also found that the level of serum FGF-21 expressed lower in healthy population and higher in high-risk AS patients and population with coronary heart disease. It may be the result of the compensatory increase of FGF-21. The positive staining rate of FGF-21 in endothelial cells of aortic arch and liver tissues in group B was higher than that of group A and group E, and that of group C and group D was lower than that of group B, which indicated that FGF-21 participated in the occurrence of AS, lipid-lowering therapy could inhibit FGF-21 activation, and exogenous FGF-21 could interpose in AS process. In early period of AS onset, the expression of various important enzymes is controlled by NF-κB during LDL modification and the formation of inflammatory medium, including 5- lipid oxidation kinase[15]. Various adhesion molecules that promote monocytes to adhere to endothelial cells and migrate to the gap under endothelium, including intercellular adhesion

Table II. Comparison of the results of immumohistochemical cases (%).

Group	Cases	Endothelial cells of aortic arch	Liver tissues
Group A	50	3 (6.0)	5 (10.0)
Group B	50	33 (66.0)	40 (80.0)
Group C	50	16 (32.0)	25 (50.0)
Group D	50	21 (42.0)	30 (60.0)
Group E	50	2 (4.0)	4 (8.0)
χ^2		64.337	82.554
p		0.000	0.000

Compared to the data before treatment, $p<0.05$.

Table III. Comparison of the results of RT-PCR method.

Group	FGF-21	NF-κB	MCP-1	MMP-9	TNF-α
Group A	0.3251±0.1023	0.2635±0.0528	0.3625±0.1025	0.4256±0.1235	0.1254±0.0529
Group B	1.5243±0.3265	0.9564±0.2154	1.1235±0.3259	1.3264±0.3265	0.6548±0.2154
Group C	1.1325±0.3152	0.6354±0.2036	0.8654±0.2458	1.0635±0.2158	0.4215±0.1326
Group D	1.1125±0.2965	0.5248±0.1258	0.7259±0.2365	0.8659±0.2633	0.4123±0.1212
Group E	0.2516±0.1125	0.2132±0.0639	0.3325±0.1126	0.4152±0.1325	0.1036±0.0326
F	42.632	25.524	36.521	52.635	16.529
p	0.000	0.000	0.000	0.000	0.000

Table IV. Comparison of the results of Western blot method.

Group	NF-κB	MCP-1	MMP-9	TNF-α
Group A	0.21±0.09	0.35±0.12	0.42±0.13	0.13±0.09
Group B	0.85±0.23	0.96±0.36	1.13±0.26	0.66±0.18
Group C	0.63±0.18	0.75±0.28	0.92±0.24	0.48±0.12
Group D	0.58±0.14	0.71±0.26	0.84±0.21	0.44±0.11
Group E	0.19±0.06	0.33±0.13	0.40±0.15	0.11±0.06
F	32.662	41.625	65.239	25.524
p	0.000	0.000	0.000	0.000

molecule intercellular adhesion molecule, accepts NF-κB activation[16]. NF-κB induces the expression of MCP-1 to enhance the ability of smooth muscle cells to adhere to monocytes and accelerate the bubblization of the taken lipoprotein in the gap of endothelium, thus to start and accelerate AS process[17]. Monocyte colony stimulating factor (M-CSF) can promote monocytes to differentiate into macrophages[18] and MMP-9 plays an essential role in macrophages' migrating to tissues as well as the degradation of extracellular matrix, which all need the induction and activation of NF-κB[19]. The occurrence of AS is a chronic and persistent inflammatory reaction process. Endothelial cells express adhesion molecules after injury to regulate the adhesion of monocytes and T lymphocytes in circulation and release various inflammatory media, including TNF-α and IL-6[20]. The results of this study showed that the levels of serum NF-κB, MCP-1, MMP-9 and TNF-α in group B were higher than those of group A and group E, and those of group C and group D were lower than those of group B. The levels of NF-κB, MCP-1, MMP-9, TNF-αmRNA and protein in endothelial cells of aortic arch and liver tissues in group B were higher than those of group A and group E, and those of group C and group D were lower than those of group B. The differences had statistical significance.

Conclusions

We observed that FGF-21 participates in the occurrence of AS, which may be related to the activation of NF-κB pathway. Lipid-lowering therapy can inhibit the activation of FGF-21 and NF-κB. Exogenous FGF-21 can also lower the activation of NF-κB and interpose in AS process.

Conflict of interest
The authors declare no conflicts of interest.

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