

Original Article

Function of basic fibroblast growth factor in native arteriovenous fistula stenosis of hemodialysis patients

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Abstract: Objective: The aim of this study was to investigate and discuss the functions and mechanisms of basic fibroblast growth factor (bFGF) in native arteriovenous fistula stenosis (AVFS) of hemodialysis patients. Methods: A total of 24 patients with stage 5 chronic kidney disease, needing vascular access reconstruction due to AVFS, were enrolled as AVFS group. Vessel segments with AVFS were collected during the reconstruction. Another eight patients receiving vascular resection because of peripheral vascular diseases were selected as the control group. General information of the patients, such as minimum inner diameter of blood vessel, age, gender, albumin (ALB), hemoglobin (Hb), and cholesterol (CHO) were collected. Fluorescent quantitative polymerase chain reaction was applied to detect messenger ribonucleic acid (mRNA) expression of α -smooth muscle actin (α -SMA) and bFGF in the blood vessels of the two groups of patients. Protein expression of transforming growth factor- β 1 (TGF- β 1) and Smad3 in the blood vessels of both groups of patients were measured via Western blotting. Results: The minimum inner diameter of blood vessel of patients in the AVFS group was remarkably smaller than that in the control group ($t=46.260$, $P<0.001$), but differences in other general information (age ($t=1.093$, $P=0.283$), male ($\chi^2=0.169$, $P=0.681$), ALB ($t=0.578$, $P=0.568$), Hb ($t=1.678$, $P=0.104$) and CHO ($t=0.509$, $P=0.615$)) were not statistically significant. In the AVFS group, mRNA expression of α -SMA ($t=7.943$, $P<0.001$) and bFGF ($t=8.384$, $P<0.001$) in patient blood vessels were elevated markedly compared with those in the control group. The same was true for protein expression of TGF- β 1 ($t=3.249$, $P=0.003$) and Smad3 ($t=3.855$, $P=0.001$). Conclusion: Massive proliferation of smooth muscle cells exists in vessel segments of AVFS hemodialysis patients. This may be associated with an increase in bFGF expression and activation of TGF- β 1/Smad3 signaling pathways.

Keywords: Native arteriovenous fistula stenosis, fibroblast growth factor, transforming growth factor-beta 1

Introduction

Native arteriovenous fistulas and other vascular accesses are the "lifelines" of hemodialysis. However, as the time after establishment of native arteriovenous fistulas is extended, intimal hyperplasia may occur in the anastomotic vessels, triggering vascular stenosis and ultimately leading to failure of hemodialysis [1-4]. Large quantities of vascular smooth muscle cells with positive alpha-smooth muscle actin (α -SMA) are distributed in the portion of vascular intimal hyperplasia. This is the major pathological manifestation of vascular intimal hyperplasia and stenosis [5, 6]. One study revealed that basic fibroblast growth factor (bFGF) could promote the proliferation and migration of vascular smooth muscle cells, thereby participat-

ing in the occurrence and development of vascular remodeling and other diseases [7]. For instance, Li et al. discovered that injured vascular endothelial cells were capable of secreting a large amount of bFGF which bound to receptors on the vascular smooth muscle cells, causing cell proliferation [8]. However, whether bFGF exerts biological effects on arteriovenous fistula stenosis (AVFS) of hemodialysis patients still remains unknown. In addition, bFGF usually exerts its biological effects via the transforming growth factor-beta 1 (TGF- β 1)/Smad3 signaling pathway. For example, Chen et al. manifested that bFGF activated TGF- β 1/Smad3 signaling pathways, thereby accelerating proliferation and differentiation of fibroblast cells and secretion of collagens [9].

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Table 1. Specific sequences of primers

Gene	Forward primer	Reverse primer
α -SMA	5'TGGCTACTCCTTCGTGACCA3'	5'GCCGACTCCATACCGATGAA3'
bFGF	5'AGCGGCTGTACTGCAAAAAC3'	5'AGCCAGGTAACGGTTAGCAC3'
GAPDH	5'CCTCTGACTTCAACAGCGACA3'	5'TGGTCCAGGGGTCTTACTCC3'

Note: α -SMA, α -smooth muscle actin; bFGF, basic fibroblast growth factor.

As a result, the α -SMA expressions in the blood vessels with AVFS were detected in this paper, and then the changes in bFGF and TGF- β 1/Smad3 signaling pathway in the AVFS of hemodialysis patients were investigated.

Materials and methods

Specimen acquisition

A total of 24 patients with stage 5 chronic kidney disease (CKD), needing vascular access reconstruction due to AVFS, were enrolled as the AVFS group. Vessel segments with AVFS were collected during the reconstruction. Another eight patients receiving vascular resection because of peripheral vascular diseases were selected as the control group.

Inclusion criteria: Patients with stage 5 CKD (glomerular filtration rate <15 mL/min), patients undergoing arteriovenous fistula operations (cephalic vein-radial artery end-to-side anastomosis), and patients with AVFS (decreased vascular murmur detected through auscultation, flow rate of hemodialysis <150 mL/min, and minimum inner diameter around the fistula \leq 1.80 mm measured via digital subtraction angiography).

Exclusion criteria: Patients with peripheral vascular thrombus or stenosis induced by such reasons as arteriovenous malformation and atrial fibrillation and patients complicated with tumors, systemic lupus erythematosus, infections, or other serious systemic diseases.

General information of the patients, including minimum inner diameter of blood vessel, age, gender, albumin (ALB), hemoglobin (Hb), and cholesterol (CHO) were collected. Signed informed consent was obtained from all enrolled patients. This research was approved by the Ethics Committee of Ji'nan Central Hospital Affiliated to Shandong University.

Detection of TGF- β 1 and Smad3 protein expression in the blood vessels via Western blotting

Vascular tissue testing methods for the two groups of patients were the same. The specific steps were as follows: After

the blood in obtained vascular tissues was washed away using ice-cold phosphate-buffered saline, the vascular tissues were added into a tissue grinder placed on the ice. They were then ground after addition of phosphate-buffered saline containing 1% protease inhibitor (Sigma, USA). Next, the protein in the slurry was quantified by bicinchoninic acid (Beyotime, China). The samples with equivalent content of total proteins were fetched for polyacrylamide gel electrophoresis. When the bromophenol blue almost reached the bottom of the gel, electrophoresis was stopped. Polyacrylamide gel was transferred to a membrane at a constant voltage of 100 V for 2 hours and the proteins were transferred to a polyvinylidene fluoride membrane. After sealing with 3% bovine serum albumin (Solarbio, China) at room temperature for 2 hours, the proteins were incubated with primary antibodies (anti-TGF- β 1 antibody (1:1,000, Abcam, USA), anti-Smad3 antibody (1:500, Abcam, USA), and anti-GAPDH antibody (1:1,000, Abcam, USA)) at 4°C overnight. Afterward, the membrane washed 3 times. Horseradish peroxidase-labeled goat-anti-rabbit secondary antibody (1:2,500, Boster, USA) was added and incubated at room temperature for 1 hours. The membrane was then washed 3 more times. This was followed by color development with an enhanced chemiluminescence developer (Beyotime, China) and exposure and photographing under a gel imager.

Detection of messenger ribonucleic acid (mRNA) expression of α -SMA and bFGF in the blood vessels via fluorescent quantitative polymerase chain reaction (PCR)

The same method was applied to examine vascular tissues of the two groups of patients. Detailed procedures are set forth below. The mRNAs in vascular tissues were extracted by TRIzol Reagent (Invitrogen). Next, mRNAs were reverse-transcribed into complementary deoxyribonucleic acids using reverse transcription

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Table 2. Comparison of general information between the two groups of patients

General information	Age (year)	Male (case)	MIDBV	ALB (mmol/L)	Hb (g/L)	CHO (mmol/L)
Control group (n=8)	51.98±2.43	5	3.38±0.16	37.53±4.40	119.06±8.75	4.51±2.33
AVFS group (n=24)	53.21±2.85	13	1.41±0.08	36.41±4.85	113.49±7.93	5.07±2.80
t/ χ^2	1.093	0.169	46.260	0.578	1.678	0.509
P	0.283	0.681	<0.001	0.568	0.104	0.615

Note: MIDBV, the minimum inner diameter of blood vessel; ALB, albumin; Hb, hemoglobin; CHO, cholesterol; AVFS, arteriovenous fistula stenosis.

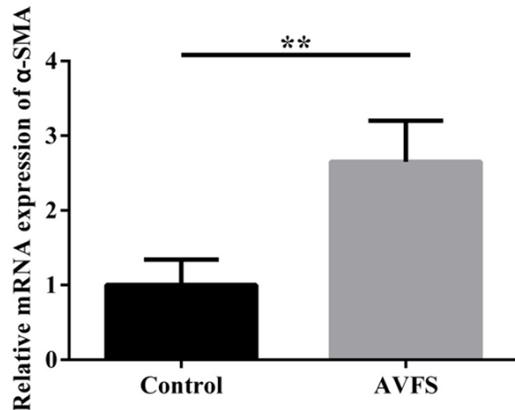


Figure 1. Comparison of α -SMA mRNA expression in blood vessels between the two groups of patients. AVFS group vs. control group, ** $P < 0.01$. α -SMA, α -smooth muscle actin; AVFS, arteriovenous fistula stenosis.

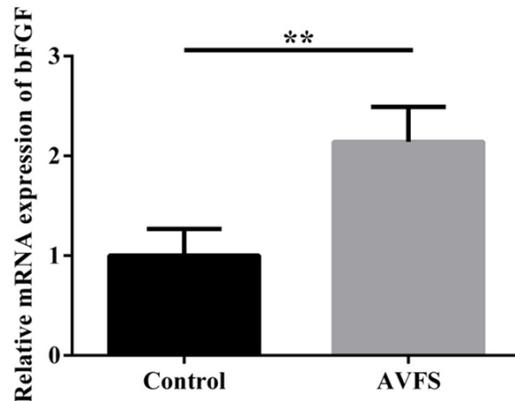


Figure 2. Comparison of bFGF mRNA expression in blood vessels between the two groups of patients. AVFS group vs. control group, ** $P < 0.01$. bFGF, basic fibroblast growth factor; AVFS, arteriovenous fistula stenosis.

kit (PrimeScript™ RT reagent Kit, TaKaRa). Finally, SYBR Mixture (With ROX, Biomiga, USA) was applied for PCR. Reaction conditions: pre-denaturation at 95°C for 10 minutes, denatur-

ation at 95°C for 15 seconds, annealing and extension at 60°C for 1 minute, for a total of 40 cycles. Primers were synthesized by Sangon Biotech (Shanghai) Co., Ltd. Specific sequences are shown in **Table 1**.

Statistical analysis

SPSS 13.0 software was utilized for statistical analysis. Measurement data are expressed as mean \pm standard deviation ($\bar{x} \pm sd$) and t-tests were performed for comparisons of data between the two groups. Image-Pro Plus 6.0 software was applied to determine the gray scale value on the Western blot picture. The gray scale value in the control group was set as 1 for comparisons. $P < 0.05$ suggests that differences are statistically significant.

Results

Comparison of general information between the two groups of patients

Minimum inner diameter of blood vessels of patients in the AVFS group was remarkably smaller than the control group ($t = 46.260$, $P < 0.001$). However, differences in age ($t = 1.093$, $P = 0.283$), male ($\chi^2 = 0.169$, $P = 0.681$), ALB ($t = 0.578$, $P = 0.568$), Hb ($t = 1.678$, $P = 0.104$), and CHO ($t = 0.509$, $P = 0.615$) were not statistically significant. All data were comparable as shown in **Table 2**.

Comparison of proliferation of vascular smooth muscle cells between the two groups of patients

In the AVFS group, α -SMA mRNA expression was elevated markedly compared to the control group ($t = 7.943$, $P < 0.001$), indicating that massive proliferation of smooth muscle cells existed in the vessel segments with AVFS as shown in **Figure 1**.

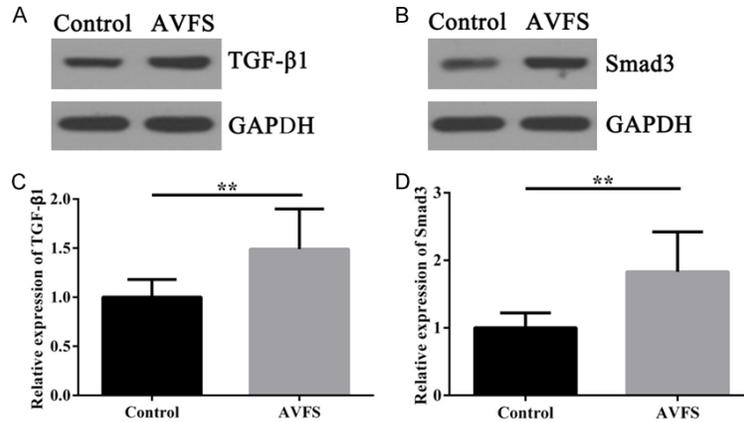


Figure 3. Comparison of TGF-β1 and Smad3 protein expression in blood vessels between the two groups of patients. A: Detection of TGF-β1 protein expression in blood vessels via Western blot; B: Detection of Smad3 protein in blood vessels via Western blot; C: Statistical analysis of TGF-β1 protein expression in blood vessels; D: Statistical analysis of Smad3 protein in blood vessels. AVFS group vs. control group, **P<0.01. AVFS, arteriovenous fistula stenosis; TGF-β1, transforming growth factor-β1.

Comparison of bFGF expression in blood vessels between the two groups of patients

bFGF mRNA expression in the blood vessels of patients in the AVFS group was notably higher than the control group (t=8.384, P<0.001), suggesting that bFGF may exert important promoting effects on proliferation of smooth muscle cells in vessel segments with AVFS as shown in **Figure 2**.

Comparison of TGF-β1 and Smad3 expression in blood vessels between the two groups of patients

Compared to the control group, patients in the AVFS group had significantly increased expression of TGF-β1 protein (t=3.249, P=0.003) and Smad3 protein (t=3.855, P=0.001) in the blood vessels (**Figure 3**), manifesting that activation of TGF-β1-Smad3 signaling pathways may be involved in the mechanism of bFGF promoting the proliferation of smooth muscle cells.

Discussion

Vascular intimal hyperplasia-induced stenosis is the most common reason for failure of vascular access, mainly pathologically manifested as distribution of massive vascular smooth muscle cells with positive α-SMA and scattered inflammatory cells at the portion of vascular intimal hyperplasia [5, 6]. For this study, fluorescent quantitative PCR was conducted to

measure α-SMA mRNA expression in blood vessels. Results indicated that such expression in patients of the AVFS group was increased significantly compared to the control group, suggesting that smooth muscle cells in vessel segments with AVFS proliferated greatly.

Since bFGF possesses such biological functions as promoting angiogenesis, repressing cell apoptosis, and inducing cell migration, it is extensively involved in many physiological and pathological processes, including organ development, tissue regeneration, and injury repair. However, it has been discovered that bFGF can participate in tissue hyperplasia and fibrosis by means of regulating proliferation and migration of vascular smooth muscle cells [9].

A study by Zhou et al. on cigarette smoke-induced pulmonary vascular remodeling in rats indicated that mRNA and protein expression of bFGF increased markedly in pulmonary artery smooth muscle cells. Results of correlation analysis manifested that the thickness of pulmonary vessel wall had a close relation with bFGF high expression [10]. Wu et al. coupled bFGF of a series of concentrations on heparinized slides, inducing the migration of vascular smooth muscle cells. They discovered that cell migration velocity increased along with elevated bFGF concentration, reaching the peak at a density of 83 ng/cm². Such a bFGF-induced migration effect has been associated with fibroblast growth factor receptor expression on vascular smooth muscle cell surfaces and its downstream migrating protein expression [11]. Although it has been proven through large quantities of disease models that bFGF promotes proliferation and migration of vascular smooth muscle cells, whether bFGF is implicated in the proliferation and migration of smooth muscle cells in vessels with AVFS has not been clarified yet. Therefore, bFGF expression was determined by fluorescent quantitative PCR for this present study. Results revealed that bFGF mRNA expression in the blood vessels of patients in the AVFS group was notably higher than the control group, indicating that bFGF

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may provide crucial promoting effects on proliferation of smooth muscle cells in vessel segments with AVFS.

TGF- β 1 is widely involved in multiple biological functions, including cell growth, differentiation, migration, extracellular matrix secretion, and immunoregulation. With artery injuries, it is secreted in a large amount to inhibit expression of matrix metalloproteinases and enhance expression of protease inhibitors, promoting the secretion of collagens and ultimately resulting in vascular intimal hyperplasia [12-14]. TGF- β 1 genes or proteins intraperitoneally injected to blood vessels can stimulate the proliferation of smooth muscle cells and promote vascular intimal hyperplasia. However, application of TGF- β 1 antibodies, soluble receptor molecules, and oligonucleotides to block TGF- β 1 signaling pathways can reduce vascular intimal hyperplasia and postpone vascular remodeling [15]. Studies have shown that TGF- β 1 is able to activate receptors on the surface of vascular smooth muscle cells, trigger phosphorylation of Smad3, and form complexes with Smad2 and Smad4, which are transferred into the nucleus to regulate expression of relevant genes [16, 17]. Consequently, Smad3 exerts key functions in TGF- β 1-mediated vascular intimal hyperplasia. For example, Edlin et al. analyzed stenotic vessel segments and discovered that expression of Smad3 and positive α -SMA were increased notably in vascular smooth muscle cells. After transfection of Smad3 genes and adenoviruses that interfere, RNA was performed in human aortic smooth muscle cells, respectively. It was revealed that Smad3 overexpression could accelerate cell proliferation, while suppressed Smad3 expression could prevent cell proliferation [18]. In this study, Western blot was conducted to detect expression of TGF- β 1 and Smad3 in the blood vessels. Results demonstrated that protein expression of TGF- β 1 and Smad3 in patient blood vessels were elevated obviously in the AVFS group, illustrating that activation of TGF- β 1/Smad3 signaling pathways is possibly implicated in bFGF-promoted proliferation mechanisms of vascular smooth muscle cells.

It has been reported that bFGF generally plays biological roles via TGF- β 1/Smad3 signaling pathways. Chen et al. elaborated that bFGF was capable of activating TGF- β 1/Smad3 signaling pathways, facilitating the fibroblast cells to secrete type I collagen. Treatment with bFGF

antisense oligodeoxynucleotides could lower proliferation, differentiation, and secretion functions of fibroblast cells. This was correlated with repression of TGF- β 1/Smad3 signaling pathway activation [9]. Yum et al. illustrated that bFGF could stimulate autocrine and paracrine of TGF- β 1 from macrophages, thereby participating in airway remodeling [19]. In addition, bFGF has been found to be involved in proliferative effects of TGF- β 1. Strutz et al. utilized TGF- β 1 to stimulate fibroblast cells. Afterward, bFGF mRNA and protein expression were increased significantly. However, the anti-bFGF antibody or bFGF receptor tyrosine kinase inhibitor could relieve proliferative effects of the fibroblast cells mediated by TGF- β 1 [20]. This suggests that bFGF interacts with TGF- β 1/Smad3 signaling pathways, jointly participating in AVFS progression. Nevertheless, the mechanisms of interaction between bFGF and TGF- β 1/Smad3 signaling pathways could not be elaborated by the results of this present study. Further studies with specific stimulation and blocking on *in vitro* cell models are necessary.

However, there are several limitations to this present study. Since researched vascular tissues were only taken from AVFS patients, specific blocking of bFGF, TGF- β 1, Smad3, and other molecules were not performed. Variations in corresponding biological effects were not observed. The relationship between bFGF and TGF- β 1/Smad3 signaling pathways was not illuminated. bFGF's involvement in the migration, differentiation, apoptosis, and other biological mechanisms of vascular smooth muscle cells was not investigated. As a result, in the next step, smooth muscle cells will be isolated from the vessel wall with AVFS for culturing. This will be processed with bFGF to observe its impacts on biological phenomena like cell proliferation, migration, differentiation, apoptosis, and TGF- β 1/Smad3 signaling pathway. Next, bFGF, TGF- β 1, and Smad3 will be specifically blocked to observe changes in corresponding biological phenomena and signaling pathways.

In conclusion, mass proliferation of smooth muscle cells in blood vessels with AVFS was preliminarily explored. This may be associated with an increase in bFGF expression and activation of TGF- β 1/Smad3 signaling pathways.

Disclosure of conflict of interest

None.

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