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Original Article

Effects of basic fibroblast growth factor (*bFGF*) on the expression of sorbitol dehydrogenase (*SDH*) in rat mesangial cells and anti-Thy-1 nephritis

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Abstract: Objective: To investigate the effect of basic fibroblast growth factor (*bFGF*) on the expression of sorbitol dehydrogenase (*SDH*) in rat mesangial cells (MsCs) and rat anti-Thy-1 glomerulonephritis (ATG) model. Methods: We conducted an *in-vitro* MsCs culture, and then stimulated MsCs with *bFGF*. A rat ATG model was also established with 24 male SD clean rats. RT-qPCR and Western Blot were applied to detect the expression of *bFGF* and *SDH* both in MsCs and in ATG model. The expression of *bFGF* and *SDH* in ATG model was also detected by hematoxylin and eosin (HE) and immunohistochemistry staining. Results: With exogenous *bFGF* stimulated, *SDH* expression in MsCs increased in time and dose dependent manner. In the ATG model, the expression of *bFGF* and *SDH* upregulated at both mRNA and protein levels with the course of nephritis prolonged. Conclusion: *bFGF* can induce the increase of *SDH* expression, and *SDH* may be involved in the development of nephritis.

Keywords: *bFGF*, anti-Thy-1 nephritis, *SDH*, mesangial cells, nephritis

Introduction

Anti-Thy-1 nephritis is a well-acknowledged model for mesangial proliferative glomerulonephritis. The disease, featured with acute mesangiolysis is followed by early inflammatory cell infiltration, mesangial proliferation and accumulation of mesangial matrix [1]. Biological functions including cytokine action, stress, cell proliferation and apoptosis regulate these changes through pathogenesis during the process of anti-Thy-1 nephritis [2-5]. Various proteins have been pointed out to be involved in mediating these biological functions. For instance, Sasaki *et al.* demonstrated that Galectin-3 regulated rat mesangial matrix synthesis and cell proliferation during laboratorial glomerulonephritis [6]. Porst *et al.* have pointed out that fibrillin-1 can modulate rat mesangial cell proliferation and even migration in anti-Thy-1 nephritis [7]. However, these studies were limited to a relatively small number of proteins, and therefore it is imperative to study more proteins involved with the regulation of the biological functions in the anti-Thy-1 nephritis.

Basic fibroblast growth factor (*bFGF*) is the prototype of the structurally related *FGF* family of proteins and is a valid chemotactic factor for fibroblasts and endothelial cells [8]. *bFGF* is expressed in a variety of cells, including endothelial cells, fibroblasts, macrophages and vascular smooth muscle cells. Due to the lack of a signal sequence characteristic of secreted proteins, the mode of cellular *bFGF* release is unclear. In addition, the effects of *bFGF* on collagen production are not clear yet. Previous studies have indicated that *bFGF* suppresses collagen synthesis *in vitro* [9]. The association of *bFGF* expression with proliferative fibrogenesis was initially presented in patients with Dupuytrein's contracture in 1992 [10]. Frank *et al.* have presented that *bFGF* expression is upregulated in human renal fibrogenesis [10]. Kadono *et al.* found that serum *bFGF* level was significantly higher in patients with systemic sclerosis (SSc) compared to normal control subjects and that long-term release of *bFGF* by inflamed tissue might result in excess fibrosis [11]. Although *bFGF* has been isolated from whole kidney homogenates, to date no studies

Table 1. The sequences of primers used in qRT-PCR

Primer		Sequence (5' to 3')
bFGF	Sense	AAGAGCGACCCACACGTCAAACTA
	Antisense	TGGACTCCAGGCGTTCAAAGAAGA
SDH	Sense	TTAGAGGAGCTGGCGTGCCTTAAA
	Antisense	TGCTTCGTCTCCTCACCCAAAGAA
GAPDH	Sense	CCACAGTCCATGCCATCAC
	Antisense	CCACCACCCTGTTGCTGTAG

bFGF: basic fibroblast growth factor; *SDH*: sorbitol dehydrogenase; GAPDH: glyceraldehyde-3-phosphate dehydrogenase, the internal reference.

have reported either the renal localization of *bFGF* or a functional role for *bFGF* in nephritis. However, *in vitro* studies conducted with the smooth glomerular mesangial cells have demonstrated the mitogenicity of bFGF in these cells [12].

Sorbitol dehydrogenase (*SDH*) is a kind of zinc-containing enzyme which catalyzes the conversion of sorbitol to fructose. *SDH* is implicated in the metabolism of various polyols and is considered to work with aldose reductase (AR) in osmotic regulation. The regulation via the polyol pathway (PP) is known to affect the accumulation of sorbitol which is implicated with diabetes mellitus and complications including neuropathy, retinopathy, and cataracts [13]. Activation of PP is a pivotal metabolic change caused by diabetic nephropathy [14]. AR was first identified as the rate-limiting enzyme in PP through reducing glucose to sorbitol [15]. Excessive accumulation of intracellular sorbitol leads to the pathogenesis of diabetic complications [16]. Many studies presented that, in addition to carbohydrates, AR can reduce lipid peroxidation-derived aldehydes as well as glutathione conjugates [17, 18]. And *SDH*, the second key enzyme in PP, catalyzes the interconversion of polyols, such as sorbitol and xylitol to their respective ketones. *SDH* deficiency leads to subsequent accumulation of sorbitol within the cell, and then contributes to diabetic complications including cataracts and microvascular abnormalities [19]. But so far, no study was conducted to elucidate how *SDH* performs in nephritis. And the present study was initially aimed to study the relationship between *bFGF* and *SDH* as well as how *SDH* affects nephritis via the model of anti-Thy-1 nephritis.

Materials and methods

Mesangial cells cultures

Mesangial cells (MsCs) from Sprague-Dawley (SD) clean rats (Chinese Academy of Sciences, Shanghai, China) weighing 150-200 g were cultivated and identified as previously reported [20, 21]. The isolated glomeruli were digested with collagen type IV (0.5%) at 37°C for 20 min. The cell density was adjusted to 10^6/mL and inoculated into 25 cm^2 culture flasks, MsCs from passages 7 to 10 were used in the experiments.

bFGF stimulation

MsCs were digested with 0.25% trypsin (containing 0.02% ethylene diamine tetraacetic acid (EDTA)) when growing to subconfluency, then seeded into 6 cm culture dishes with a cell density of 10^6/mL. When reached 80% confluency, MsCs were growth arrested for 24 h in RPMI1640 (containing 0.5% FBS). After that, human recombinant *bFGF* was added to stimulate MsCs in two ways: (1) MsCs were treated with *bFGF* of 2 ng/mL for different time (0, 12, 24 h); (2) MsCs were treated with *bFGF* of different concentrations (0, 2, 5 ng/mL) for 24 h. The total RNA and protein of MsCs were extracted with Trizol (Gibco-BRL, NY, USA) and tri-stained lysate.

Rat anti-Thy-1 glomerulonephritis (ATG) model

Twenty-four male SD clean rats weighing 150-200 g were used in rat anti-Thy-1 glomerulonephritis (ATG) and randomly allocated to two groups: (1) a normal control group (n=4); (2) a nephritis group (n=20), which were injected with 0.2 mg/kg (body weight) rabbit anti-rat Thy-1 serum into the caudal vein. The controls received an identical volume of 0.9% normal saline. After injection, renal specimens were obtained from the rats sacrificed on the 1^{th}, 3^{th}, 5^{th}, 7^{th} and 14^{th} day (n=4). The total RNA and protein were also extracted with Trizol (Gibco-BRL, NY, USA) and tri-stained lysate.

Western blot assay

Total proteins of MsCs stimulated by *bFGF* at different time points, *bFGF* of different concentrations and those extracted from ATG model at different time points (20 μg per sample) respec-

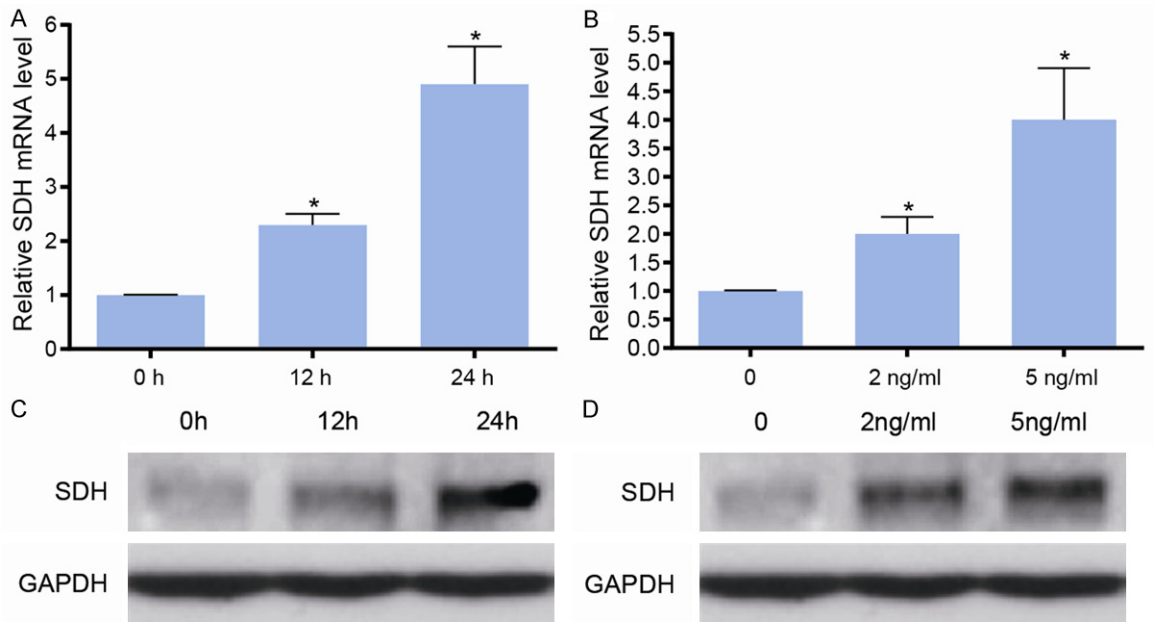

Figure 1. Expression of *SDH* in MsCs. A. MsCs were treated with *bFGF* of 2 ng/mL for different time (0, 12, 24 h); B. MsCs were treated with *bFGF* of different concentrations (0, 2, 5 ng/mL) for 24 h; **P*<0.05, versus control; C. MsCs were treated with bFGF of 2 ng/mL for different time (0, 12, 24 h); D. MsCs were treated with bFGF of different concentrations (0, 2, 5 ng/mL) for 24 h. *SDH*: sorbitol dehydrogenase; MsCs: mesangial cells; *bFGF*: basic fibroblast growth factor.

tively were separated by 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). After that, the gel was transferred to polyvinylidene fluoride (PVDF) membrane (Sigma, Steinheim, Germany). Mouse anti-human *SDH* monoclonal antibody (1:500, Santa Cruz) and rabbit anti-human *bFGF* polyclonal antibody (1:200, Santa Cruz) was selected as the primary antibody. Secondary antibody was biotinylated goat anti-rabbit (1:500, Vector), and diaminobezidin (DAB) was used as color developing agent. ImageJ software analysis (Wayne Rasband, NIH, USA) was used to analyze the stripes and corrected by the internal reference of GAPDH.

RT-qPCR

RNA samples (2 μg per sample) were respectively reversely transcribed to cDNA, including total RNAs of MsCs stimulated by *bFGF* at different time points and *bFGF* of different concentrations, and those extracted from ATG at different time points. cDNAs were equally subjected to qPCR reaction with primers showed in **Table 1**. The qPCR determination was carried out with ABI PRISM 7900HT Real-time qPCR System (Applied Biosystems, Carlsbad, CA, USA), and the relative concentration was also calculated using 2-method.

Immunohistochemistry

The kidneys of rats in ATG model sacrificed on the 7th and 14th day respectively were obtained and fixed in 4% paraformaldehyde, then embedded in paraffin, hereafter routine hematoxylin and eosin (HE) staining of 4-μm thick sections. The primary antibodies were rabbit anti-*bFGF* (1:40, Santa Cruz) and mouse anti-human *SDH* monoclonal antibody (1:300, Santa Cruz). After removal of unbound primary antibody, the sections were incubated with the secondary antibody of biotinylated goat anti-rabbit IgG (1:100, Vector), and color developing agent was DAB.

Statistical analysis

The data in this research was presented as mean ± standard deviation (SD). GraphPad Prism 6.0 (GraphPad Prism Software Inc., San Diego, CA, USA) was applied to analyze all the data. One-way ANOVA was also used to compare the multiple groups. $P<0.05$ was identified as statistical signifcance.

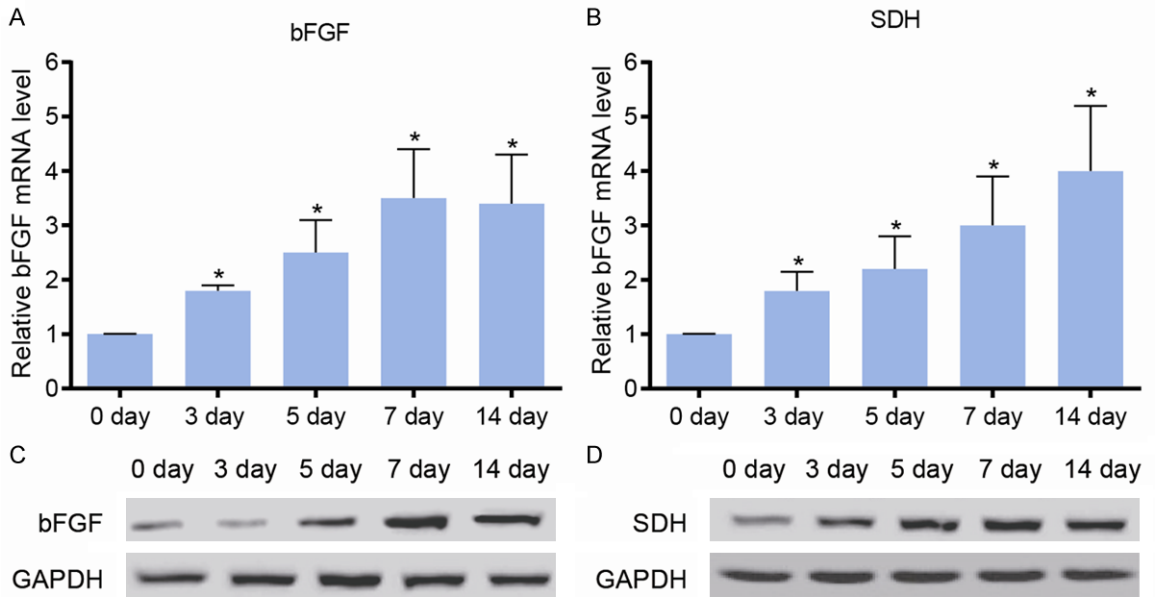

Figure 2. Expressions of *bFGF* and *SDH* in ATG tissue. A. mRNA expression of *bFGF*; B. mRNA expression of *SDH*; **P*<0.05, versus 0 day; C. Protein expression of bFGF; D. Protein expression of SDH. ATG: anti-Thy-1 glomerulonephritis; *SDH*: sorbitol dehydrogenase; *bFGF*: basic fibroblast growth factor.

Results

Effects of bFGF on expression of SDH in MsCs

The mRNA expression of *SDH* had a continuous increase after 2 ng/mL *bFGF* stimulation, and reached its peak at 24 h (*P*<0.05, **Figure 1A**). Similarly, after the treatment of *bFGF* at different concentrations, the *SDH* mRNA expression level also increased and achieved the highest at 5 ng/mL *bFGF* (*P*<0.05, **Figure 1B**). This demonstrated a time-dose dependence. The protein expression level of *SDH* was up-regulated after treated with 2 ng/mL *bFGF* and kept growth to 24 h (**Figure 1C**). With *bFGF* stimulation of different concentrations, the expression of *SDH* protein was also rising and came to the top at 5 ng/mL *bFGF*, which was consistent with the nucleic acid level (**Figure 1D**); it showed a time-dose dependence as well.

RT-qPCR and Western blot in ATG model

In the experiment of ATG model, we found that the mRNA expression of *bFGF* in renal cortical tissue was increased from day 3 after injection, and reached the top at day 7, then maintained high level until day 14 (*P*<0.05, **Figure 2A**). The mRNA expression of *SDH* continued to increase from day 3 and reached peak level at day 14 (*P*<0.05, **Figure 2B**). The protein expressions of *bFGF* and *SDH* exactly corresponded to the results of mRNA expression (**Figure 2C**, **2D**).

Immunohistochemistry in ATG model

The ATG model was confirmed to be successfully established by HE staining, immunohistochemical staining of alpha-smooth muscle actin (α-SMA) and proliferating cell nuclear antigen (PCNA). HE staining showed that MsCs proliferated to peak at 7th; MsCs in samples of 7th and 14th in nephritis group were both obviously more than that in normal control group (**Figure 3A**). In the nephritis group, *bFGF* was mainly expressed in the glomerular mesangial cells, endothelial cells and capillary wall, and a few *bFGF* positive cells were observed to appear in the renal tubular epithelial cells, whereas there was no *bFGF* found in normal renal tissue (**Figure 3B**). In normal renal tissue, *SDH* was in low expression. With the course of disease prolonged and MsCs proliferated, *SDH* began to highly express in the mesangial area and the proximal convoluted tubule epithelial cells (**Figure 3C**). In summary, the expressions of *bFGF* and *SDH* in ATG tissue were higher compared with that in normal renal tissue.

Discussion

As is indicated in the experimental results, *SDH* expression in MsCs increased in time and dose dependent manner with exogenous *bFGF* stimulated, In the ATG model, the expression of *bFGF* and *SDH* upregulated at both mRNA level

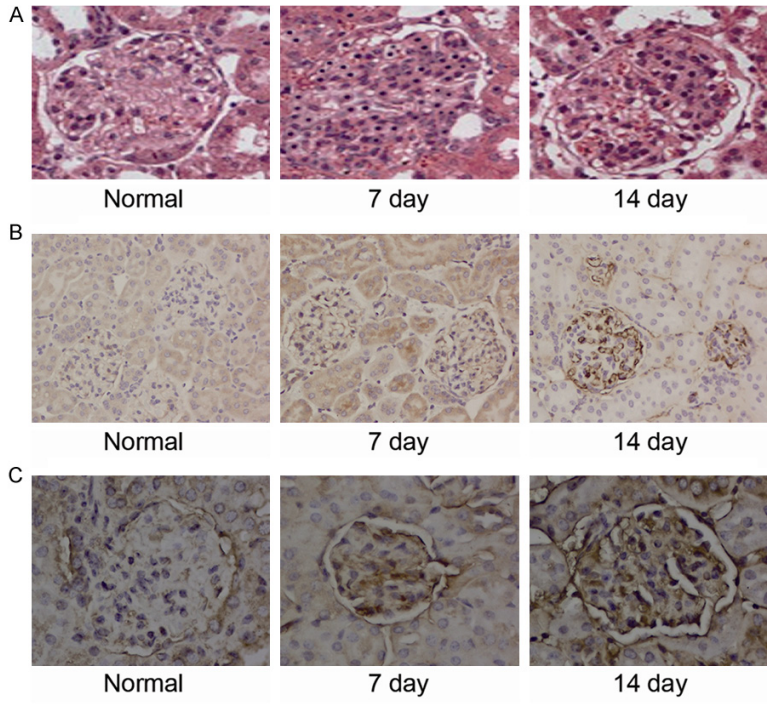

Figure 3. HE and immunohistochemistry staining in ATG model. A. HE staining; B. *bFGF* expression in glomeruli; C. *SDH* expression in glomeruli and kidney tubule; ABC staining method under 100× microscope. HE: Hematoxylin and eosin; ATG: anti-Thy-1 glomerulonephritis; ABC: avidin-biotin-enzyme compound; *bFGF*: basic fibroblast growth factor; SDH: sorbitol dehydrogenase.

and protein level with the prolongation of the course of disease. In other words, *bFGF* can promote the expression of *SDH*, and *SDH* may be involved in the pathogenesis of glomerulonephritis.

bFGF is a highly conserved 18 kDa cationic protein that belongs to the family of heparin binding growth factors [22]. It is primarily a cell-associated protein with important roles in fetal development, wound healing, neovascularization, and neuronal and smooth muscle cell growth [23]. In the kidney of fetal mouse, *bFGF* is localized in the basement membranes surrounding renal tubules and it is mitogenic for renal epithelial cells [24]. These findings suggest an important role for *bFGF* during renal tubular development. However, its mode of action in renal pathogenesis remains less clearly defined. *bFGF* released by injured mesangial cells in a rat model of immunologically-induced glomerulonephritis stimulate the proliferation of mesangial cells [25]. Furthermore, in diseases featured by glomerular epithelial proliferation such as renal and bladder tumors [26, 27], increased *bFGF* can be detected in the urine suggesting that *bFGF* can be released from cells during this process.

Previous studies have suggested that the kidney is an important site for the uptake of exogenously administered *bFGF* [28], although the renal distribution of these binding sites remains unclear. But Ray's study has demonstrated that *bFGF* binding sites were localized to renal glomeruli and vasa recta in the renal medulla [8]. Similarly, another study has presented that glomerular visceral epithelial cells (GVEC) can synthesize *bFGF* [29]. A number of glomerular cell types, such as mesangial cells [30], endothelial cells [31] and visceral epithelial cells [29], can proliferate in response to the exposure to *bFGF*. This implies that each of these cell types bears specific *FGF* receptors. *bFGF* expression was observed in epithelial cells and podocytes of Bowman's capsule of puromycin aminonucleoside nephropathy rats [32]. In the present study, there was no *bFGF* found in normal renal tissue. In the glomerulonephritis group, *bFGF* was mainly expressed in the glomerular mesangial cells, endothelial cells and capillary wall, and few *bFGF* positive cells were observed to appear in the renal tubular epithelial cells.

There are increasing experimental evidences demonstrating that the PP is involved in metabolic abnormalities. In diabetic retinopathy (DR), as the consequence of PP activation, there is an accumulation of sorbitol and fructose as well as the enhancement or generation of oxidative stress in the retina of diabetic rats and non-diabetic eye donors exposed to high glucose in cell culture [33-35]. It is well known that hyperglycemia enhances glucose metabolism via the PP. AR is the first rate-limiting enzyme in this PP, reducing glucose to sorbitol, which could be further metabolized to fructose by *SDH*, the second enzyme in the PP. It is

reported that the flux through *SDH* and up-regulated fructose level may increase advanced glycation end-products (AGEs) formation, which can contributes to diabetes-induced microvascular abnormalities [36]. As *SDH* is implicated in diabetic-associated glycation reactions, any agent able to affect the activity and/or mRNA expression of these enzymes might potentially mediate these glycation reactions [37].

Transgenic mice overexpressing AR specifically in the lenses, when they became diabetic nephropathy, presented an increase in oxidative stress significantly, as indicated by an increase of malondialdehyde and a decrease of glutathione (GSH) in their lenses. Increasing evidences in both preclinical and clinical suggest that oxidative stress plays a critical role through the pathogenesis of diabetic complications. Hyperglycemia induces free radical generation leading to lipid peroxidation, a major marker of oxidative stress. It has been reported that kidney malondialdehyde levels of diabetic rats are increased by lipid peroxidation [38]. And transferring a *SDH*-deficient mutation into these transgenic mice can significantly normalize the malondialdehyde and GSH levels. And these results show that all enzymes of the PP contribute to hyperglycemia-caused oxidative stress in the lens [39]. *SDH* deficiency leads to subsequent accumulation of sorbitol within the cell, which finally results in diabetic complications such as cataracts and microvascular abnormalities [19]. As demonstrated in the present study, in the ATG, with the course of disease prolonged and mesangial cell proliferated, *SDH* began to highly express in the mesangial area and the proximal convoluted tubule epithelial cells; whereas in normal renal tissue, *SDH* was in low expression. Furthermore, with exogenous *bFGF* stimulated, *SDH* expression in MsCs increased in time and dose dependent manner.

In conclusion, *bFGF* can induce the increase of *SDH* expression both *in vitro* and *in vivo*, and the increased *SDH* may be involved in the development of non diabetic nephropathy, which presents a new researching direction and generates a new therapy for nephritis.

Disclosure of conflict of interest

None.

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