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Effect of bone marrow mesenchymal stem cells on the Smad expression of hepatic fibrosis rats

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ABSTRACT

Objective: To investigate the impact of bone marrow mesenchymal stem cells on Smad expression of hepatic fibrosis rats. **Methods:** A total of 48 adult female SD rats were randomly divided into three groups, normal control group (n=10), observation group (n=19) with liver fibrosis model rats injected with BMSCs cells; model group (n=19), with liver fibrosis model rats injected with physiological saline. Serum index, TGF-β1 and Smad expression were detected. **Results:** Type Ⅲ procollagen, Ⅳ collagen, hyaluronic acid, laminin levels of observation group were significantly lower than those of model group (P<0.05). The content and expression of TGF-β1 in serum and liver tissue of observation group were significantly lower than those of model group(P<0.05). Compared with normal control group, the Smad3, Smad4 mRNA and protein expression of model group were significantly increased, the Smad7 mRNA and protein expression were significantly reduced (P<0.05). Compared with model group, Smad3, Smad4 mRNA and protein expression of observation group were significantly reduced, and Smad7 mRNA expression were significantly increased (P<0.05). **Conclusions:** BMSCs can regulate Smad expression to some extent, and reduce the degree of liver fibrosis.

1. Introduction

Bone mesenchymal stem cell (BMSCs) is able to differentiate into endoderm hepatic cells which can resist liver fibrosis and protect liver cells. Transplantation of exogenous BMSCs can treat hepatic inheritance metabolic diseases to some extent. Liver fibrosis is the intermediate by which a variety of chronic liver diseases develop into cirrhosis. It has been reported that in recent years that BMSCs transplantation has a good effect on the treatment of liver fibrosis. It can solve donor organ shortage for bio-artificial liver transplantation and hepatocyte transplantation, and also has a great significance in controlling the liver fibrosis[1-3].

However, a variety of factors in the surrounding environment will influence and determine the BMSCs differentiation direction and lead to the unstable therapeutic effects. In this paper, we studied the impact of BMSCs on the Smad expression levels of liver fibrosis rats and explored their relationship with collagen deposition in the live, to analyze the possible molecular mechanisms of liver fibrosis in rats from the molecular level.

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2. Materials and methods

2.1. Animal model and grouping

Totally 50 adult female clean grade SD rats were selected, 8 to 10 weeks old, weighting 80-300 g. All were purchased from Shanghai SLAC Laboratory Animal Co. Rats took food and water freely in experimental period. Ten rats were used as normal control group, the rest 40 rats as liver fibrosis model group. The model was established by CCl_4 sc method. 300 mL/L CCl_4 paraffin oil solution was injected intraperitoneally by 3 mL/kg, 2 times/w for 8 weeks, and two rats died during the experiment. A total of 38 CCl_4 treated SD rats were randomly divided into the observation group (n=19): 0.2 mL suspension with BMSCs cell suspension (containing 5×10^6 cells) were given by tail vein injection at

the first 6, 8 w of the modeling; the model group 19 (n=19): equivalent normal saline were given by tail vein injection at the first 6, 8 w of the modeling; All SD rats were sacrificed after 10 w modeling of the liver fibrosis and the liver tissue samples were obtained for detection.

2.2. Reagents and instruments

Lymphocyte separation medium (Shanghai Hua Jing Reagent Company), L-DMEM medium (Gibco, USA). Smad3, Smad7 and β-actin antibody (Santa Cruz, USA), PCR primers (Sangon, Shanghai), SYBR Green Mix and reverse transcription kit (TOYOBO Corporation, Japan), TGF β 1 immunohistochemistry kit, corollary diluent (1:100) and DAB chromogenic agent (all purchased from Wuhan Boster Biological Engineering Company), fetal bovine serum (Tianjin Hao Yang companies). JEM-1220 transmission electron microscope, YLN-2000 gel imaging analysis system, Immunohistochemistry kit: TGFlB, smad2, 3 (batch BAl396); Smad7 (batch BAl399); corollary diluent (1:100) (batch SAl022); DAB chromogenic reagent were all purchased from Wuhan Boster Biological Engineering Co.

2.3. BMSCs isolation and culture

Young male SD rats were collected, marrow suspension were isolated from long bone of limbs after 10% chloral hydrate anesthesia and routine disinfection. Interface layer cells were by absorbed density gradient centrifugation, and washed in L-DMEM culture medium twice. Cells were collected and inoculated with $1\times10^5/cm^2$ in 25 cm^2 flasks, and added with L-DMEM medium which contained 100 U/mL streptomycin, 100 U/mL penicillin and 10% fetal bovine serum. They were cultured in 37℃, 5% CO_2 incubator. Attachment culture method was used in BMSCs isolation and purification. The cells were proliferated.

2.4. Methods

2.4.1. Tissues sample collection

Blood was extracted by extirpating eyealls, and then rats were sacrificed. The blood was centrifuged, and storage in the refrigerator. One left lobe and one right lobe of liver were obtained, and fixed in 10% neutral formalin. Routine paraffin section and HE staining were performed then the tissue was observed under the light microscopic.

2.4.2. Serum index detection

Type Ⅲ procollagen, Ⅳ collagen, hyaluronic acid, laminin were detected by radioimmunoassay strictly according to the instruction of kits.

2.4.3. TGF-β 1 detection

Serum TGF-β 1 levels was measured by double antibody sandwich method. Immunohistochemistry (SP method) was used to observe liver TGF-β 1 distribution. Color extent and range were analyzed by Olympus Bx50 image analyzer. Ten good high power fields of each specimen were selected, and brown colored particles were positive staining cells. The color degree was 1 (weak), 2 (medium), 3 (strong), and the color range was in accordance with the percentage of high-power field, 1(<25%), 2 (25%-50%), 3(50%-75%), 4(>75%). The color rendering index = color degree×color range.

2.4.4. Smad detection

PCR detection: Liver tissue total RNA was extrected by TRIzol instructions. Transcription the mRNA was reversed to cDNA for quantitative PCR reaction. Smad3 upstream primer: 5′-CCAGTGCTACCTCCAGTGTr-3′, downstream: 5′-CT-GGTGGTCGCTAGnTrCTC -3′; Smad7 upstream: 5′-GGCTITCAGATFCCCAACTrC-3′, downstream 5′-CGCCATCCACTTC-CCTFGT-3′; internal reference β-actin upstream: 5′-CGTTGACATCCGTA-AAGACCTC-3′, downstream: 5′-rAGGAGCCAGGGCAGTAATCT-3′. Western blot detection method was used to extract total protein of liver tissue, then rabbit anti-mouse TGF-β 1 antibody (1:200) primary antibody and HRP-conjugated goat anti-rabbit secondary antibody (1:500) binding reaction was carried out after electrophoresis and transmembrane. They were incubated avoid light at room temperature for 1 h. The membrane was washed to detect β-actin expression as an internal reference. Fluorescence system scan was used and the gray integral value of Smad3, Smad7 protein bands was recorded. The ratio of samples integral value and internal reference integral value was used for statistical analysis.

2.5. Statistical analysis

All data were analyzed with SPSS 16.0 software. Data were expressed as mean±SD values, the difference between two groups were compared with t test. P<0.05 was considered as statistical significant difference.

3. Results

3.1. Type Ⅲ procollagen, Ⅳ collagen, hyaluronic acid and laminin

Type Ⅲ procollagen, Ⅳ collagen, hyaluronic acid and laminin in the observation group and model group were significantly higher than the normal control group (P<0.05). Type Ⅲ procollagen, Ⅳ collagen, hyaluronic acid and laminin of the observation group were significantly lower than the model group (P<0.05) (Table 1).

3.2. TGF-β 1 level

The contents and the expression of TGF-β 1 levels in serum and liver tissue of the observation group and model group were significantly higher than the normal control group (P<0.05); The contents and the expression of TGF-β 1 levels in serum and liver tissue of the observation group were significantly lower than the model group (P<0.05) (Table 2).

3.3. Smad3, Smad4 mRNA expression

Compared with the normal control group, the Smad3, Smad4 mRNA expression of the observation group and model group were significantly higher than the normal control group (P<0.05). Compared with the model group, the Smad3, Smad4 mRNA expression of the observation group were significantly reduced, the Smad7 mRNA expression was significantly

increased (P<0.05) (Table 3).

3.4. Smad3, Smad4 protein expression

Compared with the normal control group, the Smad3, Smad4 protein expression of the observation group and model group were significantly higher than the normal control group, while the Smad7 protein expression was significantly decreased (P<0.05); Compared with the model group, the observation group Smad3, Smad4 protein expression was significantly reduced, Smad7 protein expression was significantly increased (P<0.05) (Table 4).

4. Discussion

Liver fibrosis is caused by the imbalance of synthesis and degradation of extracellular matrix and the overdeposition of extracellular matrix, which is the main pathological processes from various chronic liver disease to cirrhosis. BMSCs are a class of stem cells from mesoderm with multipotential differentiation potential. Study confirmed that BMSCs can differentiate into chondrocytes, osteoblasts, myocardial cells, fat cells and other mesoderm-derived tissue cells, but also differentiate into hepatocyte-like cells and neuron-like cells from the endoderm and ectoderm[4-7]. Tai *et al*[8] transplanted human BMSCs into allyl alcohol induced liver injury rats, and obsered human hepatocyte-specific alpha-fetoprotein, AGPR, CK18, CK19 and ALB expression by immunohistochemistry. It showed human BMSCs could be induced into hepatocytes in appropriate microenvironment. We observed the effect of BMSCs on the Smad expression of liver fibrosis rats, and explored the mechanism of BMSCs liver fibrosis reversal effect at the molecular level in this study.

It has been reported that the TGF β -Smad signaling pathway can regulate the damage repair,tumor growth and metastasis and other biological behavior is also the main message pathway of liver fibrosis[9]. TGF- β 1 plays an important role in the process of liver fibrosis, while Smads protein as a key post-receptor messenger protein that mediates TGF- β 1 signal from the cell membrane to nucleus. Smad protein family is involved in the regulation cell transformation, proliferation, synthesis, secretion and apoptosis, which can be divided into three categories in accordance with the different functions: the receptor activated SMADs is the target molecules of TGF- β 1 receptor complex downstream effects; Common type Smad, such as Smad4, can combined with receptor-activated Smad and plays a role in the TGF- β 1 signal transduction as a transit molecules; inhibitory Smad, mainly includes Smad7, can inhibit the activation of receptor-activated SMADs and block the biological effects of TGF- β 1[10-13]. Studies suggest that the adjustment of key Smads molecules level expression have an effective anti-fibrotic effect which can be used as the assessment index[14].

Table 1
Comparison of hepatic serological markers detection of rats in each group (ng/mL).

Groups	Number of cases	Type Ⅲ procollagen	Type Ⅳ collagen	Hyaluronic acid	Laminin
Observation group	19	189.4±2.6*#	136.1±3.7*#	215.8±8.3*#	163.3±10.8*#
Model group	19	279.8±3.1*	179.5±4.3*	356.2±10.8*	205.7±16.6*
Normal control group	10	97.1±2.2	48.1±3.1	131.8±7.8	156.4±10.5

Note: Compared with normal control group, * P<0.05; compared with model group, # P<0.05.

Table 2
Contents and the expression of TGF- β 1 levels in serum and liver tissue of two groups.

Groups	Number of cases	Serum TGF- β 1(μg/L)	Tissue TGF- β 1
Observation group	19	0.73±0.11*#	4.36±1.28*#
Model group	19	1.31±0.15*	10.41±3.45*
Normal control group	10	0.23±0.09	1.00±0.00

Note: Compared with normal control group, * P <0.05; compared with model group, # P <0.05.

Table 3
Comparison of Smad3, Smad4 mRNA expression of two groups

Groups	Number of cases	Smad3	Smad4	Smad7
Observation group	19	1.48±0.07*#	1.22±0.06*#	0.86±0.09*#
Model group	19	2.13±0.09*	1.53±0.10*	0.41±0.07*
Normal control group	10	1.03±0.06	1.01±0.08	1.00±0.05

Note: Compared with normal control group, * P<0.05; compared with model group, # P<0.05.

Table 4
Comparison of Smad3, Smad4 protein expression of two groups.

Groups	Number of cases	Smad3	Smad4	Smad7
Observation group	19	0.50±0.06#	0.82±0.18#	0.99±0.13#
Model group	19	0.82±0.10*	1.25±0.12*	0.51±0.10*
Normal control group	10	0.25±0.08	0.91±0.12	1.06±0.16

Note: Compared with normal control group, * P <0.05; compared with model group, # P<0.05.

Most reports showed that BMSCs can reduce the liver fibrosis. Liang *et al*[15] found that after 4 weeks of BMSCs transplantation for the treatment of CCl_4-induced hepatic fibrosis in rats, the degree of liver fibrosis can be significantly reduced. Zhang *et al*[16] found that the level of fibrosis in the hepatic lobule was significantly decreased after BMSCs transplantation. Zhao[17] found that the serum hyaluronic acid, serum type IV collagen and serum type III procollagen levels of the mesenchymal stem cells treatment group were significantly lower than the model group. This study detected the liver serological markers of rat and found that the liver tissue structure has been significantly improved after BMSCs transplantation. The number of false lobe was significantly reduced and the fibrous septa turned thinner. Ⅲ procollagen, Ⅳ collagen, hyaluronic acid, laminin level of the observation group were significantly higher than the normal control group. Although the observation group Ⅲ procollagen, Ⅳ collagen, hyaluronic acid, laminin level is still significantly higher than the normal control group, the collagen fibers in the observation group was significantly reduced compared with the model group, the hyaluronic acid, laminin levels were also significantly reduced. It showed that BMSCs can block the excessive production and deposition of hepatic collagen or other extracellular matrix,mitigate and control liver fibrosis, also have the degradation effect on the formed extracellular matrix.

The results also show that Smad3, Smad4 mRNA and protein expression levels of the model group were significantly increased, the difference was significant compared with the normal control group($P<0.05$), which showed the Smad3, Smad4 expression levels were increased significantly during rat experimental hepatic fibrosis formation. The Smad3, Smad4 mRNA and protein expression levels of the observation group were significantly lower than the model group, which showed that BMSCs can inhibit the Smad3, Smad4 expression to some extent. Xing *et al*[18] considered that Smad3-mediated most of the pro-fibrotic effects of TGF β. Transgenic rat Liver type Ⅰ collagen reduced with the decrease of liver endogenous Smad4 expression, which showed the inhibition of Smad3, Smad4 expression has a potential anti-fibrotic effect. Smad7 is an antagonistic protein which is able to tightly bind with T β RIV, so the binding of Smad2 or Smad3 with T β RIV were blocked by Smad7, and the Smad2 or Smad3 phosphorylation were inhibited[19]. By this way, Smad7 can complete the inhibition of TGF- β signal transduction. Subeq *et al*[20] injected the Smad7cDNA transfection adenovirus vector to fibrosis rats and found that α-smooth muscle actin and liver collagen expression both decreased. In this study, the Smad7 mRNA and protein expression of the observation group were significantly higher than the model group, which showed that BMSCs can increase the expression of Smad7 and reduce fibrosis from another point of view.

In summary, injection of BMSCs into the vail vein for liver fibrosis rat can significantly improve liver histological status. BMSCs can regulate the expression of Smad to some extent, and provide a new method chronic liver fibrosis and other fibrotic diseases.

Conflict of interest statement

We declare that we have no conflict of interest.

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