Role of the Notch Signaling Pathway in Fibrosis of Denervated Skeletal Muscle

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Summary: In order to investigate the role of the Notch signaling pathway in skeletal muscle fibrosis after nerve injury, 60 Sprague-Dawley rats were selected and divided randomly into a control and two experimental groups. Group A served as controls without any treatment. Rats in groups B were injected intraperitoneally with 0.2 mL PBS and those in group C were injected intraperitoneally with 0.2 mL PBS+100 μmol/L, 0.2 mL N-[N-(3,5-difluorophenacetyl)-l-alanyl]-S-phenylglycine t-butyl ester (DAPT, a gamma-secretase inhibitor that suppresses Notch signaling) respectively, on postoperative days 1, 3, 7, 10, and 14 in a model of denervation-induced skeletal muscle fibrosis by right sciatic nerve transection. Five rats from each group were euthanized on postoperative days 1, 7, 14, and 28 to collect the right gastrocnemii, and hematoxylin and eosin (HE) staining, immunohistochemistry test, real-time PCR, and Western blotting were performed to assess connective tissue hyperplasia and fibroblast density as well as expression of Notch 1, Jagged 1, and Notch downstream molecules Hes 1 and collagen I (COL I) on day 28. There was no significant difference in HE-stained fibroblast density between group B and C on postoperative day 1. However, fibroblast density was significantly higher in group B than in group C on postoperative days 7, 14, and 28. Notch 1, Jagged 1, Hes 1, and COL I proteins in the gastrocnemius were expressed at very low levels in group A but at high levels in group B. Expression levels of these proteins were significantly lower in group C than in group B (P<0.05), but they were higher in group C than in group A (P<0.05) on postoperative day 28. We are led to conclude that locking the Notch signaling pathway inhibits fibrosis progression of denervated skeletal muscle. Thus, it may be a new approach for treatment of fibrosis of denervated skeletal muscle.
Key words: Notch signaling pathway; sciatic nerve; skeletal muscle fibrosis; N-[N-(3,5-difluorophenacetyl)-l-alanyl]-S-phenylglycine t-butyl ester; Notch 1; Jagged 1; Hes 1; collagen I; denervated muscular atrophy

Peripheral nerve injury is one of the most common clinical traumas with limited treatment and poor therapeutic outcomes that have always been a major problem in clinical medicine[1–3]. Nerve regeneration is relatively slow, and denervated skeletal muscle undergoes muscular atrophy, fibrosis, and degeneration at relatively rapid rates. Skeletal muscle often loses the capacity for myocyte regeneration before regaining nerve control, which leads to irreversible skeletal muscle atrophy[4, 5] and greatly impairs functional recovery of the injured peripheral nerve. Thus, prevention and reduction of skeletal muscular atrophy before the skeletal muscle regains innervation is key to ensuring functional rehabilitation after nerve repair. In recent years, many studies have shown that overexpression of the Notch signaling pathway leads to scleroderma[6], spontaneous pulmonary fibrosis[7], renal fibrosis[8], myocardial fibrosis[9], and other diseases. Hence, this study established a denervated skeletal muscle model and applied a gamma-secretase inhibitor, N-[N-(3,5-difluorophenacetyl)-l-alanyl]-S-phenylglycine t-butyl ester (DAPT), to suppress Notch signaling. This was followed by assessing expression of Notch signaling pathway-related molecules Notch 1, Jagged 1, Hes 1, and collagen I (COL I) using immunohistochemistry, real-time PCR, and Western blotting as well as examining skeletal muscle morphology using hematoxylin and eosin (HE) staining to determine

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the relationship between Notch signaling and skeletal muscle fibrosis.

1 MATERIALS AND METHODS

1.1 Experimental Materials

The experimental protocol was approved by the Ethics Committee on Animal Experimentation at our University. Animal experiments were carried out strictly in accordance with the manual of animal experiments. The number of rats used for the experiments was minimized to fulfill the experimental needs.

1.1.1 Experimental Animals Sixty specific pathogen-free healthy male Sprague-Dawley rats (4–5 months of age, weighing approximately 200–250 g) were provided by the Experimental Animal Center of Tongji Medical College, Huazhong University of Science and Technology [Animal license number: SCXK (Hubei Province) 2008-0004]. Animal experiments were carried out at the Experimental Animal Center of Tongji Medical College, Huazhong University of Science and Technology. The animals were fed and housed in an environment with relative humidity set to 50% and temperature maintained at 18–25°C. All rats were divided randomly into three groups (Groups A, B, and C) with 20 animals per group.

1.1.2 Major Instruments and Reagents Major experimental instruments and reagents used in this study included an inverted phase contrast microscope (CKX41-A22PHP, Olympus, Japan), a real-time fluorescence-based quantitative PCR machine (Applied Biosystems 2720 Thermal Cycler, Thermo Fisher Scientific Corp, USA), DAPT (Alexis Biochemicals, USA), rabbit anti-rat Notch antibody, rabbit anti-rat Jagged 1 antibody, rabbit anti-rat Hes 1 antibody, rabbit anti-rat COL I antibody, and goat anti-rabbit secondary antibody (Abcam, Cambridge, UK).

1.2 Experimental Methods

Experimental rats were weighed and then anesthetized with 1% pentobarbital sodium (35 mg/kg) via intraperitoneal injection. This was followed by shaving the hair in the surgical field (right hip and right lower limb), fixation on a small animal experimental platform, conventional disinfection, and surgical draping. Under aseptic conditions, an oblique skin incision was made on the right hip to expose the gluteus maximus and biceps femoris, and this was followed by blunt dissection. The sciatic nerve was carefully freed from the space between the gluteus maximus and biceps femoris, and 1 cm of length was exposed. In group A (control group), the sciatic nerve was exposed without any treatment. In groups B and C, the sciatic nerve was cut at 1 cm from the lower edge of the piriformis, and 1 cm of the sciatic nerve was removed. This was followed by complete hemostasis, rinsing the wound twice with normal saline, closing the intermuscular groove, and suturing the skin incision with 4-0 surgical silk thread. The animals were kept warm and monitored after the surgery. They were then housed and fed in separate cages after recovery. The rats in group B were injected intraperitoneally with 0.2 mL PBS, and the rats in group C were injected intraperitoneally with 0.2 mL PBS and 0.2 mL,100 μmol/L phenylglycine (DAPT, to suppress Notch signaling), respectively, at the same sites on postoperative days 1, 3, 7, 10, and 14. Five rats from each group were euthanized by cervical dislocation on postoperative days 1, 7, 14, and 28 to harvest the right gastrocnemius for later experiments.

1.3 Indicator Detection

1.3.1 Histomorphological Analyses HE staining was performed to observe connective tissue hyperplasia and fibroblast growth. After conventional deparaffinization and rehydration, tissue sections were immersed in distilled water three times, stained with hematoxylin for 10 min, and excess dye was removed by rinsing with water, and the blue was enhanced by immersing the slides in tap water for 20 min. This was followed by eosin staining for 2 min, ethanol dehydration, xylene clearing, and mounting with neutral balsam. Five visual fields from each slide were selected randomly to assess average fibroblast density (number of fibroblasts per square millimeter) under light microscope.

Immunohistochemistry was used to detect expression of Notch 1, Jagged 1, Hes 1, and COL I proteins in the gastrocnemius. After conventional deparaffinization and rehydration, tissue sections were washed three times with PBS. Antigen retrieval was performed by adding sodium citrate droplets and incubating the sections in a 37°C water bath for 10 min. After three washes with PBS, tissue sections were blocked for 1 h. After three washes with PBS, 40 μL rabbit anti-rat Notch 1, rabbit anti-rat Jagged 1, rabbit anti-rat Hes 1, and rabbit anti-rat COL I antibodies (1:150 dilution) were added separately to the tissue sections and incubated overnight in a moist chamber at 4°C. After three washes with PBS, goat anti-rabbit secondary antibody was added to the tissue sections and incubated at 37°C for 1 h. After washing with PBS, 3,3′ diaminobenzidine (DAB) reagent was used for color development, which was terminated by immersing in tap water. Hematoxylin dye was used for counter stain of nuclei. Five non-overlapping high quality visual fields (×200 magnification) were selected randomly for imaging. Image-Pro Plus 6.0 (Beijing Daheng Image Vision Co., Ltd., China) image analysis software was used to determine the absorbance (*A*) value of positive reactants in each image and to perform statistical analysis and comparisons.

1.3.2 Measurement of Notch 1, Jagged 1, Hes 1, and COL I mRNA levels Real-time quantitative PCR was used to detect mRNA expression of Notch

1, Jagged 1, Hes 1, and COL I using the following reaction conditions: 95°C initial denaturation for 2 min; 45 cycles of 95°C denaturation for 15 s, 55°C annealing for 15 s, and 72°C elongation for 45 s. SYBR Green I fluorescent dye was used for labeling to obtain a standard curve of each group of specimens and to calculate Ct values for computer analysis.

1.3.3 Expression of Notch 1, Jagged 1, Hes 1, and COL I Proteins Western blotting was used to detect Notch 1, Jagged 1, Hes 1, and COL I protein expression in 20 mg rat tissue samples from each group. Total protein of each sample was isolated from a tissue homogenate, separated in a protein gel, and transferred to a nitrocellulose membrane. After blocking the membrane, the corresponding anti-rat Notch 1, anti-rat Jagged 1, anti-rat Hes 1, and anti-rat COL I antibodies (1:400) were incubated separately with the membrane overnight at 4°C. After three washes in Tris buffered saline with Tween (TBST), horseradish peroxidase-labeled secondary antibody (1:2400) was incubated with the membrane at room temperature on a slow shaker for 30 min. This was followed by washing the membrane in TBST solution three times on a shaker and placing the membrane in a gel visualization system (Media Cybernetics, USA) for analysis and imaging.

1.4 Statistical Analysis

SPSS 17.0 software (SPSS Inc., USA) was used for statistical analysis. All data are presented as mean±standard deviation ($\bar{x}\pm s$). One-way analysis of variance was used to compare data between groups. $P<0.05$ was considered statistically significant.

2 RESULTS

2.1 HE Staining Results

HE staining showed no significant changes in morphology of gastrocnemii in groups B and C on postoperative day 1. Both groups showed myocytes with normal morphology and orderly arrangement, and these features were not significantly different from those of group A. On postoperative day 7, connective tissue of gastrocnemius bundles in group B began to show hyperplasia with some fibroblast infiltration. In contrast, connective tissue of gastrocnemius bundles in group C showed less initial hyperplasia and fibroblast content. On postoperative day 14, myocytes of the gastrocnemius in group B were disordered with significant connective tissue hyperplasia and a large number of infiltrated fibroblasts. In contrast, pathological changes in group C were inhibited and less severe. On postoperative day 28, myocytes of the gastrocnemius in group B were severely disordered with significant connective tissue hyperplasia and maximal fibroblast infiltration. By comparison, degrees of connective tissue hyperplasia and fibroblast infiltration of the gastrocnemius in group C were much lower (fig. 1). Table 1 presents the degrees of fibroblast infiltration in the different groups.

2.2 Immunohistochemistry Results

In group A, immunohistochemistry of gastrocnemius specimens on postoperative day 28 showed relatively weak Notch 1, Jagged 1, Hes 1, and COL I protein expression in the extracellular matrix. After inducing sciatic nerve injury, high protein expression levels of

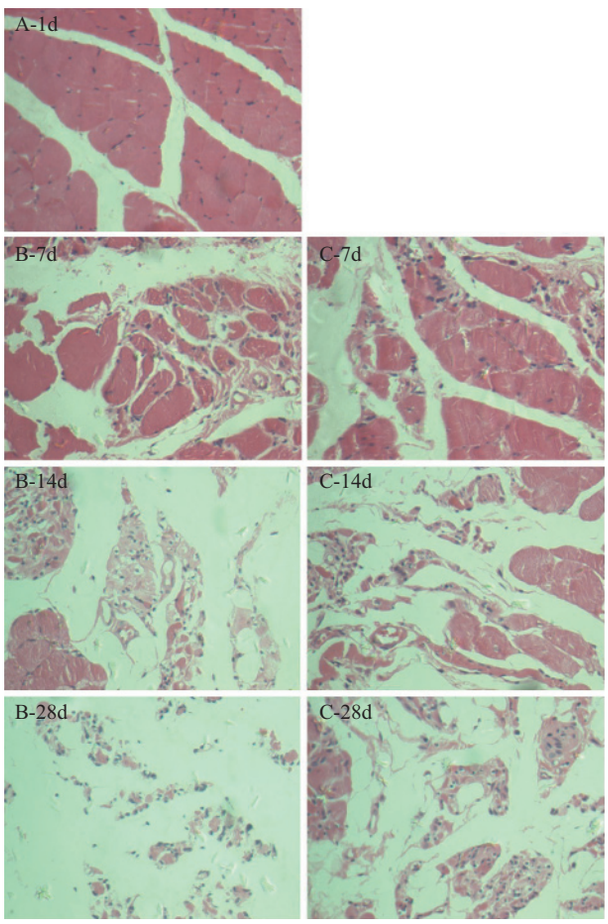

Fig. 1 HE staining of gastrocnemii under inverted phase contrast microscope (×200)
Morphological changes of gastrocnemii in groups B and C were not apparent on postoperative day 1. Myocytes showed normal morphology and orderly arrangement, and no significant morphological differences were found between groups A, B, and C. On postoperative days 7, 14, and 28, gastrocnemius muscle bundles in group B began to exhibit hyperplasia with fibroblast infiltration. The degree of hyperplasia was proportional to duration following nerve resection. Increases in connective tissue hyperplasia and fibroblast infiltration in the gastrocnemius muscles of group C were less pronounced than those of group B. A, B, C: group A, B, C; d: days after operation

Table 1 Fibroblast densities in groups B and C ($\times 10^3$, $\bar{x}\pm s$)

Groups	Day 1	Day 7	Day 14	Day 28
B	1.27±0.52	2.14±0.82*	2.98±0.74*	3.84±1.31*
C	1.21±0.46	1.63±0.79*	2.35±0.62*	2.91±0.96*

*Significant difference between groups B and C, $P<0.05$

Notch 1, Jagged 1, Hes 1, and COL I were found in group B (PBS-treated group). In contrast, Notch 1, Jagged 1, Hes 1, and COL I protein expression levels in group C (DAPT-treated group) were significantly reduced but still higher than those in group A (fig. 2). Table 2 shows the average *A* values of different proteins detected by immunohistochemistry in the different groups and indicates statistically significant differences between groups ($P<0.05$).

2.3 Notch 1, Jagged 1, Hes 1, and COL I mRNA Expression

Results of quantitative RT-PCR showed that after applying DAPT to inhibit Notch signaling in the rat model of skeletal muscle fibrosis after sciatic nerve transection, expression of different signaling molecules in the Notch pathway was significantly changed. As shown in fig. 3, sciatic nerve transection significantly elevated mRNA expression of Notch 1, Jagged 1, Hes

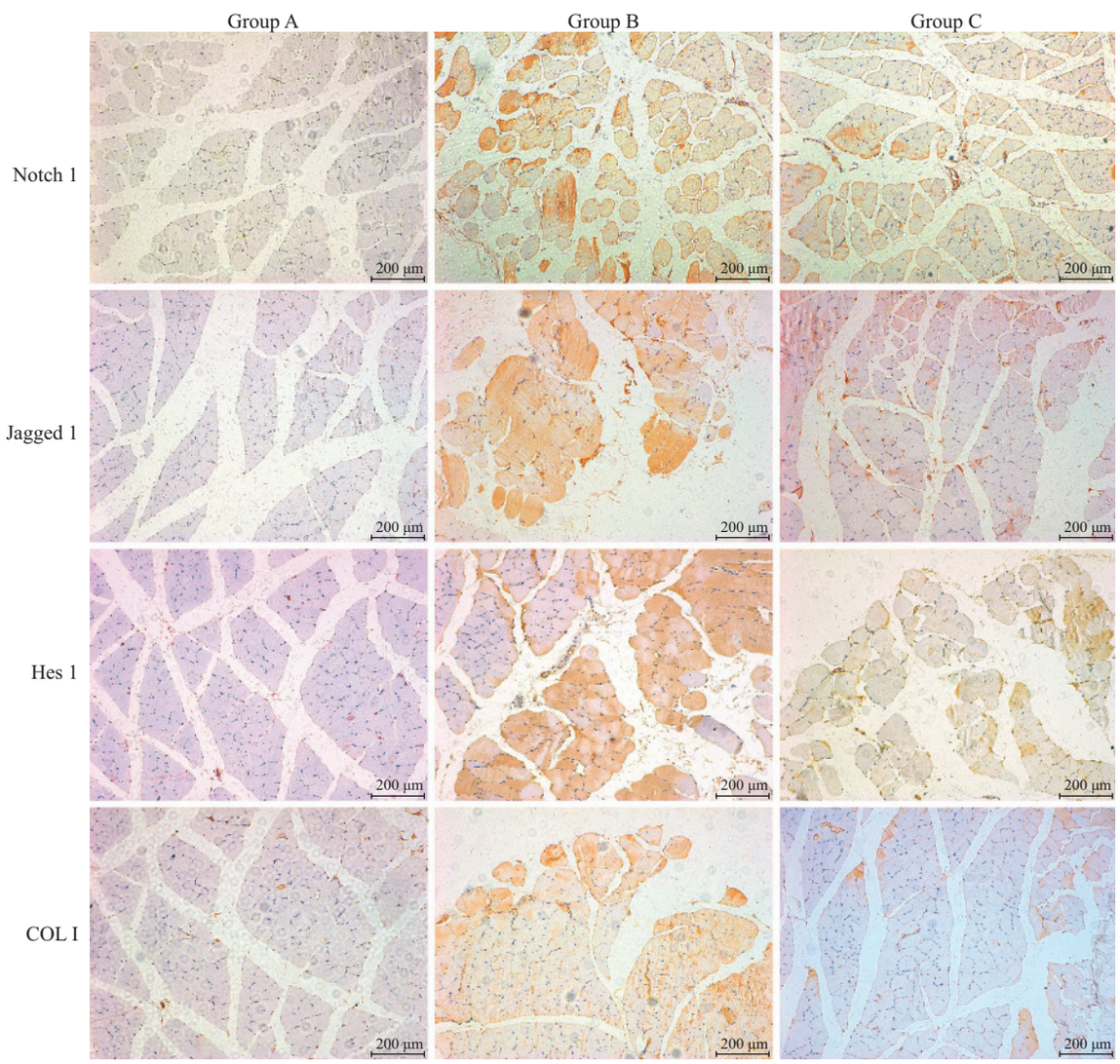

Fig. 2 Immunohistochemical detection of Notch 1, Jagged 1, Hes 1, and COL I protein expression in gastrocnemii on postoperative day 28
In group A, signals for Notch 1, Jagged 1, Hes 1, and COL I were weak in the extracellular matrix, indicating low expression of these proteins. In contrast, immunohistochemistry revealed strong signals in group B, which suggests high protein expression levels. Finally, signals were much lower in group C than in group B, but higher than in group A, indicating that DAPT reduces expression of Notch 1, Jagged 1, Hes 1, and COL I but not quite to baseline levels.

Table 2 Average absorbance value of different proteins on postoperative day 28 measured by immunohistochemistry ($\bar{x}\pm s$)

Groups	Notch 1	Jagged 1	Hes 1	COL I
A	0.1328±0.0316	0.1731±0.0274	0.2152±0.0374	0.1412±0.0194
B	0.7366±0.0384*	0.8558±0.0258*	0.7935±0.0628*	0.6833±0.0584*
C	0.4176±0.0159†,‡	0.5220±0.0263†,‡	0.4146±0.0318†,‡	0.3811±0.0471†,‡

*$P<0.05$ *vs.* group A; †$P<0.05$ *vs.* group B; ‡$P<0.05$ *vs.* group A

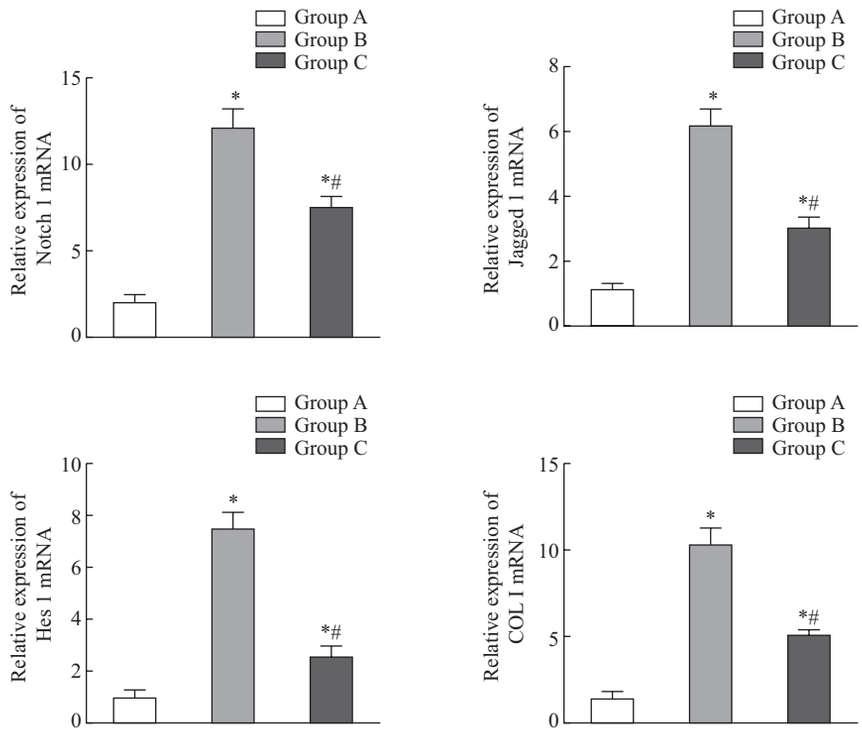

Fig. 3 Notch 1, Jagged 1, Hes 1, and COL I mRNA expression levels are related to the Notch signaling pathway in gastrocnemii in groups A, B, and C
Notch 1, Jagged 1, Hes 1, and COL I mRNA expression levels in the gastrocnemius muscles of group C were significantly higher than those in group A, but lower than those in group B. $^{*}P<0.05$ *vs.* group A; $^{\#}P<0.05$ *vs.* group B

1, and COL I. This suggests that injury of the sciatic nerve activates Notch signaling in skeletal muscles, thereby increasing expression of different signaling molecules in the Notch pathway. Application of DAPT to inhibit Notch signaling significantly reduced mRNA expression of Notch 1, Jagged 1, Hes 1, and COL I in rats with sciatic nerve transection; however, expression levels of these mRNAs were still increased as compared with the normal control group.

2.4 Notch 1, Jagged 1, Hes 1, and COL I Protein Expression in Gastrocnemii

As shown in fig. 4, Notch 1, Jagged 1, Hes 1, and COL I protein expression levels in the skeletal muscles of rats with sciatic nerve transection were elevated on postoperative day 28. This also suggests that sciatic nerve injury activates Notch signaling in skeletal muscles. In contrast, application of DAPT to inhibit Notch signaling significantly reduced Notch 1, Jagged 1, Hes 1, and COL I protein expression; however, expression of these proteins was still increased as compared with group A.

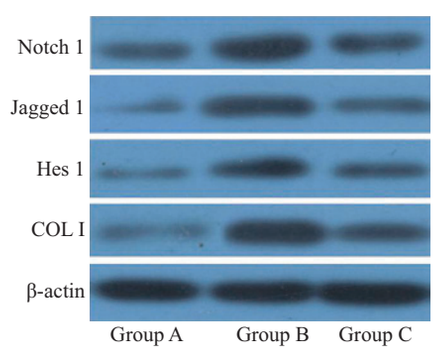

Fig. 4 Western blotting analysis of Notch 1, Jagged 1, Hes 1, and COL I protein expression levels in gastrocnemii
Notch 1, Jagged 1, Hes 1, and COL I protein expression levels were higher in group B than in groups A and C, and the protein expression levels were higher in group C than in group A.

3 DISCUSSION

Peripheral nerve injury is one of the common clinical diseases. In recent years, with development and application of microsurgical techniques, repair of injured peripheral nerves has improved, but therapeutic outcome is still not satisfactory. The main reasons for incomplete recovery of peripheral nerve function after repair of peripheral nerve injury are as follows: (1) Post-injury dominant skeletal muscle atrophies and degenerates due to loss of neurotrophic function. Long-term neurotrophic dysfunction leads to skeletal

muscle fibrosis and irreversible degeneration. (2) Peripheral nerve regeneration is relatively slow (approximately 1 mm per day). However, denervated skeletal muscles undergo relatively rapid atrophy, fibrosis, and degeneration, which results in irreversible degeneration of skeletal muscle before peripheral nerve regeneration[10]. Thus, to promote rehabilitation of peripheral nerve function in patients after repairing peripheral nerve injury, the following therapeutic goals should be considered: (1) to improve the regeneration of peripheral nerves, and (2) to prevent fibrosis of skeletal muscle, which is the effector organ of the peripheral nervous system. Preventing fibrosis of skeletal muscles before they regain innervation has become an area of intense research.

Many studies have shown recently that overexpression of Notch signaling is associated with diseases such as scleroderma[11], idiopathic pulmonary fibrosis, renal fibrosis[12], and myocardial fibrosis[13]. In addition, Gazava *et al* have shown that this pathway is widely present in the tissues of vertebrate and non-vertebrate animals. These studies provide an initial basis for our hypothesis that the Notch signaling pathway plays an important role in development of fibrosis in denervated skeletal muscles. In this study, we observed that rats with sciatic nerve transection showed atrophy and fibrosis in the gastrocnemius, which had higher fibroblast densities than gastrocnemii in untreated control rats. In addition, mRNA of Notch signal transduction-related molecules (Notch 1, Jagged 1, and its downstream molecule Hes 1) and mRNA of a key indicator of organ fibrosis, COL I, were significantly overexpressed. These changes were confirmed by Western blotting, which suggests that sciatic nerve injury activates the Notch signaling pathway (increased Notch 1 and Jagged 1 expression). This in turn leads to excessive deposition of extracellular matrix, which is mainly composed of collagen. Excessive deposition of extracellular matrix is a characteristic manifestation of tissue fibrosis.

To further verify the hypothesis that Notch signaling plays an important role in development of fibrosis in denervated skeletal muscles, we injected DAPT, an inhibitor of Notch signaling, into rats with sciatic nerve injury and then assessed changes in expression of Notch signal transduction-related molecules (Notch 1, Jagged 1, and its downstream molecule Hes 1) as well as a key indicator of organ fibrosis, COL I. DAPT, a gamma-secretase inhibitor, is a specific blocker of the Notch signaling pathway, and it exerts inhibition effect in intracellular cytoplasmic region. When extracellular ligands bind to Notch, the ICD is cleaved by gamma-secretase and then transported into the nucleus to activate downstream molecules. DAPT has been used extensively to verify the relationship between Notch signaling and certain diseases such as heart disease[14], systemic scleroderma[15], wound healing[16], pulmonary fibrosis[17], liver fibrosis[18] and chronic renal disease[19].

In this study, following sciatic nerve transection, myocytes of gastrocnemii were disordered, and connective tissue hyperplasia was significant. In contrast, intraperitoneal injection of DAPT to inhibit Notch signaling in denervated gastrocnemii attenuated atrophy and fibrosis, and greatly reduced fibroblast content. In addition, DAPT injection significantly reduced mRNA expression of Notch 1, Jagged 1, Hes 1, and COL I on postoperative day 28, which suggests that DAPT inhibits Notch 1, Jagged 1 (Notch 1 ligand), Hes 1 (Jagged 1 downstream molecule), and COL I expression at the transcription level. Results of Western blotting also showed that Notch 1, Jagged 1, Hes 1, and COL I protein expression was higher in rats with sciatic nerve injury than in untreated control rats and denervated DAPT-treated rats. However, expression of these proteins was still higher in DAPT-treated rats with sciatic nerve injury than in untreated control rats. These results suggest that DAPT inhibits mRNA expression of Notch 1, Jagged 1, Hes 1, and COL I at the transcriptional level, thereby reducing corresponding protein translation. Taken together, these results confirm that activation of Notch signaling causes excessive deposition of extracellular matrix, which eventually leads to fibrosis of denervated skeletal muscle and remodeling of structural abnormalities. However, DAPT inhibits Notch signaling by lowering the biological activity of Notch to suppress COL I expression, which is the major component of the extracellular matrix, thereby inhibiting fibrosis of denervated skeletal muscle.

Although many previous studies[20] and the present study have shown that DAPT effectively blocks Notch signaling to inhibit tissue fibrosis, some studies[21] have indicated that maintenance of Notch signaling is crucial for immunity and normal gastrointestinal function. Thus, it is possible that immune and digestive functions of test subjects may be affected after DAPT block of Notch signaling. However, in this study, we did not observe any side effects of DAPT on immune and digestive functions, and no relevant research has indicated that DAPT affects immune and digestive functions of test subjects. Hence, we believe that DAPT is safe and without any adverse effects.

In conclusion, this study confirmed that activation of Notch signaling leads to fibrosis in denervated skeletal muscle, and DAPT inhibits this pathological process. Thus, inhibition of Notch signaling may provide a new treatment for fibrosis of denervated skeletal muscle.

Conflict of Interest Statement

The authors declare that there is no conflict of interest with any financial organization or corporation or individual

that can inappropriately influence this work.

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