Tacrolimus induces fibroblasts apoptosis and reduces epidural fibrosis by regulating miR-429 and its target of RhoE

Xiaolei Li 1, Hui Chen 1, Shuguang Wang, Jihang Dai, Lianqi Yan, Jingcheng Wang *, Yu Sun **

Department of Orthopedics and Orthopedic Institute, Clinical Medical College of Yangzhou University, Subei People’s Hospital of Jiangsu Province, Yangzhou 225001, China

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ABSTRACT

Tacrolimus (FK506) has been demonstrated to reduce epidural fibrosis. However, the detailed mechanism of action has not been elucidated. Aberrant miR-429 is involved in many diseases. The aim of this study was to describe the exact mechanism of FK506 induced apoptosis in fibroblasts and the prevention of epidural fibrosis. FK506 induced fibroblast apoptosis was evaluated using CCK-8 assays, flow cytometry, and western blotting. The expression of miR-429 in fibroblasts treated with FK506 was determined by RT-qPCR. Additionally, luciferase activity assays were used to determine the target relationship between miR-429 and RhoE. Flow cytometry and western blot analysis were used to determine the effects of FK506 and miR-429 on fibroblast apoptosis. The effects of FK506 and RhoE on fibroblast apoptosis were determined by CCK-8 assay, flow cytometry, and western blotting. We also evaluate the effects of FK506 and miR-429 on epidural fibrosis in rats by using histological analysis and TUNEL-staining. The results revealed FK506 induces fibroblast apoptosis and significantly downregulates miR-429 expression in fibroblasts. Additionally, miR-429 downregulation caused the apoptosis of fibroblasts. The luciferase activity assay confirmed that RhoE is a direct target of miR-429 and RhoE promotes fibroblast apoptosis. The rat model demonstrated miR-429 inhibition promotes fibroblast apoptosis and epidural fibrosis, which is consistent with the results of FK506 treatment. Our study demonstrates that FK506 induces fibroblast apoptosis and reduces epidural fibrosis by regulating miR-429 expression and its target of RhoE.

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1. Introduction

Epidural fibrosis is a major cause of failed back surgery syndrome following posterior spinal surgery. The fibrosis is caused by the formation of excessive scar tissue [1]. The fibrotic area can compress or tether the nerve roots and lead to uncomfortable complications. The possible need for a revision surgery is a major drawback because of the complications of revision surgery for epidural fibrosis, which include dural tears and nerve root injury [2].

Although the mechanism of epidural fibrosis has not been completely understood, it appears that fibroblasts proliferation plays a primary role in the formation of fibrosis. Various biological and synthetic agents are used prevent epidural fibrosis by inducing apoptosis of fibroblasts and have achieved satisfactory results [3,4]. Tacrolimus is widely used as a potent immunosuppressive agent to treat autoimmune diseases and graft rejection of organ transplantation by suppressing T-cell function [5]. This drug has recently been reported to inhibit fibroblast proliferation and induce apoptosis [6]. As a result, tacrolimus has been used to reduce subconjunctival scarring and peritoneal adhesions after intestinal transplantation and glaucoma filtration surgery [7,8]. Our previous research showed tacrolimus could prevent epidural fibrosis after laminectomy in rats. However, the detailed mechanisms describing how tacrolimus prevents epidural fibrosis have not been elucidated [9].

MicroRNAs (miRNAs) are small non-coding RNAs that regulate gene expression post-transcriptionally by repressing or cleaving targeted miRNAs [10,11]. Previous studies have shown miRNAs regulate the proliferation and apoptosis of fibroblasts [12,13]. Previous researches showed that miRNA-489 could inhibit silica-
induced pulmonary fibrosis by repressing MyD88 and Smad3 [14].

Our previous study reported tacrolimus could downregulated miR-429 expression in fibroblasts and predicted that miR-429 had binding sites within the 3’-UTR of RhoE(RND3) using different algorithms. Previous studies showed miR-429 and RhoE regulated proliferation and apoptosis of multiple cell types [15,16]. Our present research was to investigate the effect of tacrolimus on fibroblast apoptosis and epiludal fibrosis via the regulation of miR-429 and its target of RhoE.

2. Materials and methods

2.1. Fibroblast culture

The present study was approved by the Ethical Committee and Research Committee of Subei Hospital of Jiangsu Province. The human fibroblasts were purchased from Guangzhou Senino Biotech Co., Ltd. Passages 3 and 6 for all of the experiments. The fibroblasts were cultured in DMEM (Gibco, USA) supplemented with 10% fetal bovine serum (Gibco, USA), 100 U/ml penicillin G and 100 U/ml streptomycin in an atmosphere of 5% CO2 at 37 °C.

2.2. Cell viability assay

Cell counting Kit-8 (CCK-8) was used to detect cell viability after FK506 treatment (Killerhlin, Co. Kerry, Ireland). The fibroblasts were cultured in triplicate in 96-well plates. The fibroblasts were treated with 0.5 μM, 1 μM, 5 μM, 10 μM, 25 μM, 50 μM, 75 μM and 100 μM FK506. The treated cells were maintained in DMEM for 12 h, 24 h, 36 h, 48 h and 60 h. The cell viability was determined according to the manufacturer’s instructions.

2.3. Analysis of annexin V/prodipidum iodide double staining

The fibroblasts were plated in 60-mm dishes (3 ml, 1 × 10⁶/well) and incubated for 24 h at 37 °C. The detached and adherent cells were harvested after washing twice with ice-cold PBS following various treatments. The cells were then resuspended in binding buffer at a concentration of 1 × 10⁶ cells/ml and incubated with Annexin V-FITC and propidium iodide (BD, Biosciences, USA). The cells were incubated in the dark for 15 min and then the apoptotic rates of the fibroblasts were determined using FACSDiva Software (Becton Dickinson, San Jose, CA).

2.4. Western blot analysis

Fibroblasts treated with various conditions were lysed in RIPA buffer (Beyotime, Hangzhou, China) according to the manufacturer’s instructions. The lysates were centrifuged at 12000 × g for 15 min. The protein supernatants were collected and the protein concentration was determined. Equal amounts of total protein were subjected to electrophoresis and then transferred to PVDF membranes. The membranes were blocked with 5% skim milk in TBST for 2 h and then incubated with the primary antibodies overnight at 4 °C. The membranes were washed three times in TBST and incubated with secondary antibodies for 2 h at 4 °C before analysis.

2.5. Cell transfection and infection

All of the human miRNA mimics, inhibitors, and their scramble controls were purchased from Ribo Biological Co., Ltd (Ribo, Guangzhou, China). We obtained 100 nM and 200 nM miR-429 mimics and inhibitors according to the manufacturer’s instructions by using Lipofectamine 2000 (Invitrogen, CA, USA). The lentiviral vector pLV-RhoE-inhibitor and the packaging plasmid mix were obtained from GeneChem Co. Ltd (Shanghai, China). The individual plasmids and the packaging mix were coinfected into HEK293TN packaging cell lines using a commercial reagent (Invitrogen). The culture supernatant was concentrated using Lentipacketiviruses concentration solution (Genecompany, Guangzhou, China). The control plasmids provided with the lentivirus kit were processed as described above to obtain the control pseudovirus. Fibroblasts were incubated with the lentiviruses for at least 96 h to select stably transfected cells.

2.6. miRNA target prediction

We used three databases to predict the downstream targets of the miRNAs. The three databases were TargetScan, microRNA.org, and PITA. A target was considered positive if it was predicted by at least two of the three algorithms.

2.7. Generation of RhoE 3’-UTR reporter constructs and luciferase activity assays

The RhoE 3’-UTR reporter constructs were generated by Pingkang Biological Co., Ltd. (Pingkang, Shenzhen, China). Briefly, the cDNA fragment of the human RhoE mRNA 3’-UTR containing the predicted miR-429 target site was obtained by RT-PCR and cloned into the pLUC-Reporter (Ambion, Inc., Austin, TX, USA) downstream of the luciferase coding sequence. We created two mutant constructs containing seven nucleotide mutations at the predicted miR-429 binding site region (1584–1591 or1729–1735) in the human RhoE mRNA 3’-UTR using the PCR mutagenesis method. HEK 293T cells were seeded in 24-well plates at a density of 2 × 10⁴ cells/well and then cotransfected with control or miR-429 mimics and either the pLUC-Reporter plasmid or the pRL-TK internal control plasmid (Promega, Madison, WI, USA). The luciferase activities were measured using the Dual-Luciferase Reporter Assay System (Promega) according to the manufacturer’s instructions 24 h after transfection.

2.8. Quantitative real-time PCR (qRT-PCR)

The quantitative real-time PCR was performed using the Applied Biosystems 7300HT machine and MaximaTM SYBR Green/ROX qPCR Master Mix (Fermentas, USA). The stem-loop reverse-transcription (RT) primers were designed and purchased from Nanjing Jiancheng Bio. Ins(Jiangsu China). The PCR reaction was evaluated by melting curve analysis and the PCR products were analyzed on 2% agarose gels. GAPDH amplification was used as an internal control. The data were processed using the 2^−△△Ct method.

2.9. Rat model and groups

72 healthy male SD rats were used in present study and the study was approved by The Animal Ethics Committee of Yangzhou University. The 72 rats were randomly divided into the following six groups (twelve rats in each group): FK506, Lv-miR-429, Lv-anti-miR-429, FK506 + Lv-anti-miR-429, FK506 + Lv-miR-429, and control groups.

The rat model was established according to previous studies [9]. All the procedures were performed carefully to protect the neural tissue. We applied cotton pads soaked with 0.5 mg/ml FK506 or saline to the laminectomy defects for 5 min in the corresponding groups after satisfactory hemostasis was achieved. All surgical incisions were carefully closed. The Lv-miR-429 and Lv-anti-miR-429 (0.1 ml) were injected into the center of the laminectomy defects on weeks 1, 2, and 3 in the corresponding groups.
Fig. 1. FK506 application for 24 h could induce the apoptosis of fibroblasts. (A-B) CCK-8 was applied to detect cell viability after treatment with different concentrations and various durations. FK506 inhibit the growth of fibroblast in both a dose-dependent and time-dependent manner. (C-D) Cell apoptosis was detected by using Annexin V/Propidium Iodide double staining. Fibroblasts were treated with 50 μM FK506 for various durations. The results of Flow cytometry and apoptosis rates are shown in bold and bar graphs with the results of the statistical analysis. (E-F) Fibroblasts were stained with TUNEL for 24 h after FK506 treatment. Blue indicates cell nuclei and green is the TUNEL staining. (G) The western blot results showed that FK506 could increase the expression level of apoptosis proteins (cleavage PARP and bax/bcl-2). β-actin was used as a control. Gels were run in triplicate. The histograms in panels represent the mean ± S.E.M of three independent experiments. *P < 0.05, compared with 0 or the control group. #P < 0.05 compared with 25 μM FK506 group. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)
2.10. Histological analysis

The remaining six rats in each group were used for histological analysis after four weeks. The whole L1 spinal column was removed en bloc with the surrounding paraspinal muscles and epidural fibrotic tissue. The tissues were then immersed in 10% buffered formalin for 1 week. The specimens were embedded in paraffin after decalcification with EDTA. Successive 4-μm transverse sections were made through the L1 vertebra and the sections were stained with hematoxylin-eosin (H&E).

2.11. TUNEL staining of epidural scar tissue

TUNEL staining was performed to determine the apoptotic rate of fibroblasts in the epidural scar tissue. The photographs were captured by a fluorescent microscope (Zeus) and merged using Java-based imaging software. The percentage of TUNEL-positive cells was calculated as the number of TUNEL-stained cells divided by the number of DAPI-stained cells. The cells were counted by an observer who was blinded to the experiment.

2.12. Statistical analysis

All results are expressed as means ± S.E.M. The analyses were conducted using SPSS software (version 13.0). All P-values <0.05 were considered statistically significant.

3. Results

3.1. FK506 inhibits cell viability and induces fibroblast apoptosis

The results of CCK-8 assay showed that FK506 inhibited cell proliferation and reduced the viability of fibroblasts. Our results also showed the cell viability reached a relatively minimal level at 100 μM FK506. The results of CCK-8 assays showed that FK506 (50 μM) treatment reduced cell viability with prolonged stimulation. Thus, FK506 inhibits fibroblast viability in vitro and the inhibitory effect is both dose-dependent and time-dependent. The AV/PI assay and TUNEL staining showed that FK506 induced fibroblast apoptosis in a time-dependent and dose-dependent manner. The fibroblasts treated with different doses of FK506 for 24 h showed increased apoptosis proteins levels (cleaved PARP and bcl-2/Bax) by western blot. Cumulatively, our results demonstrated FK506 reduces cell viability and induces apoptosis in fibroblasts (Fig. 1).

3.2. FK506 could downregulate the expression of miR-429 and RhoE is a target gene of miR-429

The results of RT-qPCR were described in Fig. 2A, the levels of miR-429 in 25 μM and 50 μM FK506-treated groups were significantly lower than the control group. Moreover, the level of miR-429 in 50 μM FK506-treated group was significantly lower than the 25 μM FK506-treated group. These results indicate FK506 down-regulates miR-429 expression in fibroblasts.

As shown in Fig. 2B, the target binding site regions of miR-429 proposed to be in the regions 1584 to 1591 and 1729 to 1735 of RhoE. As shown in Fig. 2C, miR-429 dramatically decreased the relative luciferase activity in HEK293T cells transfected with wild-type RhoE 3′-UTR reporter. However, miR-429 did not affect the luciferase activity when cells were transfected without miR-429. The luciferase activity of the mutated RhoE 3′-UTR reporter constructs demonstrated the miR-429 target-binding site regions were 1584–1591 and 1729–1735.

As shown in Fig. 3A and B, FK506 treatment and the miR-429 inhibitors enhanced RhoE expression in fibroblasts. However, treatment with miR-429 mimics suppressed the expression level of RhoE compared to the control group or cells transfected with the controls. Furthermore, miR-429 mimics could reverse the effect of FK506 on the RhoE expression level.

3.3. The effects of miR-429 and RhoE on fibroblasts apoptosis

The western blotting results showed miR-429 and FK506 increased the expression of apoptotic proteins such as bax/bcl-2 and cleaved PARP. However, the increased bax/bcl-2 and cleaved PARP caused by FK506 was partially attenuated by miR-429 mimics. Furthermore, the results of flow cytometry showed FK506-treated could downregulate miR-429 expression and
induced apoptosis of fibroblast. Conversely, the miR-429 mimics could reverse the increased apoptosis of fibroblast caused by FK506-treated. The results shown in Fig. 3A-D illustrate the downregulated miR-429 expression is caused by the FK506 treatment and can induce fibroblast apoptosis.

As it is shown in Fig. 3E-H, the western blot results illustrated that Bax expression level was decreased and the Bcl-2 expression level was increased in the RhoE knockdown group. Additionally, the CCK-8 assay results and AV/PI double staining showed that knockdown of RhoE increased the viability of fibroblasts and decreased apoptosis.

3.4. Inhibits the function of miR-429 could induce the apoptosis of fibroblast and reduce epidural fibrosis in a rat model

The representative HE staining histological pictures of epidural fibrosis in each group are shown in Fig. 4A. There was severe fibrosis around the laminectomy site in both the miR-429 and
Fig. 4. Inhibits the function of miR-429 could induce the apoptosis of fibroblast and reduce epidural fibrosis in a rat model. (A) Photographs show the loose scar tissues (asterisks) in the FK506 group and FK506 + anti-miR-429 group. Moderate scar adhesion (asterisks) was found in FK506 + miR-429 group and anti-miR-429 group. Dense scar tissue (asterisks) was found in the miR-429 group and control group. The sections were stained with hematoxylin–eosin (HE) with the magnifications of 40x. S means spinal marrow and L means laminectomy defect. (B-C) Representative sections stained with TUNEL to determine apoptosis of fibroblasts in epidural fibrosis in rats. The treatment of FK506 and the application of anti-miR-429 increased the number of TUNEL-positive fibroblasts. The application of miR-429 could partially attenuate the increased TUNEL-positive cell number caused by FK506. Statistical analysis results of TUNEL-positive cells were shown in the bar graphs. *P < 0.05 versus control group.
control groups. There was also moderate scar tissue at the laminectomy site in the FK506+miR-429 group. There was limited scar tissue observed round the laminectomy site in the FK506 and antimiR-429 groups. The FK506+anti-miR-429 group showed loose and limited scar tissue at the laminectomy site. These results indicate FK506 treatment inhibits the function of miR-429 and reduces epidural fibrosis in rats.

The representative images of TUNEL and statistical analysis are shown in Fig. 4B-C. There were a limited number of apoptotic fibroblasts observed in the miR-429 and control groups. However, there were many apoptotic fibroblasts observed in the FK506+anti-miR-429 group. We also found miR-429 could partially attenuate the increased apoptosis in fibroblasts caused by FK506. These results suggest treatment with FK506 inhibits the function of miR-429, induces the apoptosis of fibroblasts, and successfully reduces epidural fibrosis in rats.

4. Discussion

Fibroblast proliferation/hyperplasia plays an important role in the formation of epidural fibrosis. After laminectomy surgery, the fibroblasts in the bone defect sites produce collagen and extracellular matrix after being activated by inflammatory cytokines and growth factors. This activation leads to the formation of excessive fibrous connective tissues to repair the local defects and subsequently results in epidural fibrosis. Thus, the inhibition of fibroblast activities by different drugs is an effective method to reduce epidural fibrosis [17,18].

Tacrolimus is an immunosuppressive agent that is used for preventing allogeneic organ transplant by inhibiting T-lymphocyte function [19]. It also can treat severe atopic dermatitis and severe proliferating and apoptosis of various cells including T-cells, macrophages, eosinophils, and neutrophils. Previous studies have shown tacrolimus inhibits the proliferation of lung fibroblasts, conjunctival fibroblasts, and peritoneal fibroblasts cells by inducing apoptosis [7,8,21]. Tacrolimus is also used to reduce postoperative scar fibrosis in intestinal transplantation and glaucoma filtration surgery due to its effects on fibroblasts [7,8]. Our previous study demonstrated tacrolimus reduces postoperative epidural fibrosis in a rat model [9]. However, tacrolimus can suppress the immune activity and may affect the wound healing process and induce serious complications. As a result, the exact mechanism of how tacrolimus reduces epidural fibrosis after laminectomy surgery must be clarified.

Previous studies have reported many miRNAs regulate the proliferation and apoptosis of fibroblasts and played important roles in regulating the formation of fibrosis. Prior studies have shown miR-21and miR-200b can promote fibroblasts proliferation and fibrosis [22,23]. However, miR-155 can induce lung fibroblast apoptosis and prevent fibrosis [12]. Our results indicate tacrolimus significantly downregulates the expression of miR-429 in fibroblasts. MiR-429 is a member of the miR-200 family and is located on chromosome 1p36. Our data suggest the downregulation of miR-429 (tacrolimus treatment or transfection downregulation) induces apoptosis in fibroblasts and reduces epidural fibrosis by upregulating its target RhoE.

The study also showed that downregulation of miR-429 (tacrolimus treatment or transfection downregulation) induces apoptosis of fibroblasts. We examined multiple parameters to determine the effect miR-429 downregulation on fibroblast apoptosis. The apoptotic rates of fibroblasts significantly increased after the miR-429 was downregulated. The expression levels of apoptotic makers (cleaved PARP and the ratio of Bax/Bcl-2) also increased, which is consistent with the apoptotic rates. Furthermore, we found the miR-429 promoter could reverse the effect due to downregulation of miR-429. The results demonstrated that tacrolimus could induce the apoptosis of fibroblast due to the downregulation of miR-429.

We further investigated the anti-fibrosis effects of inhibiting miR-429 and tacrolimus treatment by examining epidural fibrosis in post-laminectomy rats. The results of histological analysis and TUNEL staining indicated that downregulation of miR-429 (tacrolimus treatment or transfection downregulation) reduced epidural fibrosis and increases of fibroblast apoptosis. However, miR-429 could partially attenuate the effects of tacrolimus on epidural fibrosis in rats. The results of rats were consistent with those of fibroblasts. These data suggest tacrolimus downregulates miR-429 expression. Our results also indicated that tacrolimus could successfully induce apoptosis of fibroblasts and reduce epidural fibrosis in rats by downregulating miR-429 expression.

MiR-429 exhibited its biological effects on fibroblasts by regulating its target gene expression. The luciferase activity assay results revealed that RhoE is a direct target of miR-429, and the 3′-UTR has two binding regions within miR-429. Moreover, miR-429 has adverse effects on RhoE expression. Our data indicate the overexpression of miR-429 downregulated RhoE expression. RhoE plays an important role in the organization of the actin cytoskeleton and regulates the apoptosis of several cell types. Previous studies have shown that overexpression of RhoE leads to apoptosis in many cancer cells [24,25]. Lin reported that deficiency of RND3 promotes mouse ependymal epithelia proliferation and results in aqueduct stenosis and hydrocephalus [26]. Another study reported that RhoE inhibits cell proliferation and induced apoptosis in prostate cancer [16]. Poch reported RhoE is a tumor inhibitor in glioblastoma cells because of its ability to inhibit ERK activation and induce cell apoptosis [27]. These data demonstrate that RhoE functions as a tumor suppressor gene and can induce cell apoptosis. Our study also demonstrated that downregulation of RhoE expression decreases the apoptotic rate and the expression of apoptotic makers such as Bax/Bcl-2. Moreover, the downregulation of RhoE attenuated the pro-apoptotic effect of tacrolimus. Therefore, tacrolimus induces apoptosis by regulating miR-429 and its target RhoE.

This study revealed that tacrolimus induces the apoptosis of fibroblast and reduces epidural fibrosis in rats by downregulating miR-429 and its target RhoE. We noted that tacrolimus significantly downregulates the expression level of miR-429 in fibroblasts. The downregulation of miR-429 induces the apoptosis of fibroblasts and reduces epidural fibrosis in rats. Moreover, we demonstrated RhoE is a direct target of miR-429 and plays a critical role in the apoptosis of fibroblast during tacrolimus treatment. Our findings describe the mechanism of tacrolimus in reducing epidural fibrosis and may provide a new approach to reducing epidural fibrosis following posterior spinal surgery.

Conflict of interest

The authors declare no conflict of interest.

Acknowledgments

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