Review

Hypoxia inducible factor-1α mediates the mechanism of the Hedgehog pathway in tendinopathy repair by Asperosaponin VI

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ABSTRACT

Background: Our previous study found that asperosaponin VI (ASA VI) has a positive effect on the repair of tendinopathy. However, its molecular biological mechanism is unclear.

Objective: To investigate the role of hypoxia inducible factor-1α (HIF-1α) in mediating the hedgehog (Hh) pathway in tendinopathy repair by ASA VI.

Methods: A total of 36 2-month-old female SD rats were classified into the normal group (NG, n = 10) and tendinopathy model group (MG, n = 26). The tendinopathy model group was further divided into the model group (MG), ASA VI group (AG), and triamcinolone acetonide + lidocaine group (TG).

Results: Compared with those in the MG group, IL-1 mRNA was significantly downregulated and IL-4 and IL-10 were increased in the AG group (P < 0.01). The mRNA expression levels of MMP3, TIMP3, VEGF-A, KDR, and VWF mRNA decreased (P < 0.01). Immunofluorescence staining revealed that CD31/endothulin levels were significantly attenuated. Scx, Mox, EYA1, EYA2, COL1, COL3, and TNC mRNA levels showed significant differences (P < 0.01). Immunofluorescence staining suggested the upregulation of Scx and the downregulation of Sox9, Shh, Ptc1, Smo, Gli1, Cyc-D1, Cyc-E1, and c-Myc mRNA levels were downregulated (P < 0.01). The protein expression levels of Gli 1, Shh, and Ptc1 decreased significantly (P < 0.01). The immunofluorescence staining levels of Shh, Ptc, and Gli 1 significantly decreased.

Conclusion: ASA VI inhibits local vascular hyperproliferation and downregulates the HIF-1α/Hh pathway to promote the tendinous differentiation of tendon stem/progenitor cells and the repair of tendinopathy. The effect of ASA VI on HIF-1α levels may be an effective target in the treatment of tendinopathy.

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1. Introduction

Tendons are specialized connective tissue that connects bones and muscles; tendinopathy is a common soft tissue disease, with Achilles tendon injury occurring in 2%–65% of people every year [1]. Tendinopathy is one of the predominant diseases treated by using TCM, among which Dipsacus is considered as an analgesic and anti-inflammatory drug that can supplement the liver and kidney and benefit blood vessels and bone development [2]. The pathogenesis of degenerative tendinopathy is related to several factors [3–5], including the abnormal differentiation of tendon stem/progenitor cells (TSPCs), hypoxia, ischemia, excessive mechanical stress, and inflammation [6,7]. It is often characterized by calcification, ossification, fibrosis, lipid deposition, and proteoglycan accumulation [4]. Tendon ectopic ossification is mainly mediated by the hypoxia-induced transcription factor HIF-1α and HIF-1α regulates MSC chondrogenic differentiation, chondrocyte proliferation, vascularization, and bone formation [8]. However, this factor is not tissue-specific. Hedgehog (Hh) has been shown to play an extremely important regulatory role in the abnormal differentiation of tendons, the development of cartilage and bone, and the maintenance of the proliferation and self-renewal potential of stem cells [9]. Elevated Hh signaling, through endochondral bone formation, induces the differentiation of soft tissue by ectopic ossification and subsequently participates in the progression of tendinopathy [10].

Previous studies have shown that TSPCs differentiate toward ossification under local vascular hypoproliferation stimulation, which is an important pathogenesis of tendinopathy [11]. Asperosaponin VI (ASA VI) has neuroprotective, myocardial protective, anti-osteoporosis, liver protective, and lipid-lowering effects [26]. The tendinopathy model group was established in reference to Khan et al. [15] as follows: A rat was fixed in the prone position on a small animal surgical operating table then injected with 300 ng of prostaglandin E2 in the middle of its left Achilles tendon once a week for a total of four times. After 4 weeks of prostaglandin E2 injection, two animals were randomly taken from the normal and tendinopathy model groups for HE and Masson staining to observe fiber arrangement, cell density, and inflammation and confirm whether the animal model had been successfully established. Subsequently, the tendinopathy model group was divided into the model group (MG, n = 8), ASA VI group (AG, n = 8), and triamcinolone acetonide + lidocaine group (TG, n = 8).

After 3 days of prostaglandin E2 injection, ASA VI (CAS number: 39524-08-8, purchased from Shanghai Jinye Biotechnology Co., LTD., HPLC test purity of 98%, batch number: 18BM10L83256) was administered through subcutaneous injection into the left leg at the dose of 40 mg/kg for 8 weeks for 3 days/time. The skin around the Achilles tendon was lifted, and the drug was injected into the loose connective tissue around the Achilles tendon without causing tendon tissue injury. Triamcinolone acetonide (Zhejiang Xianju Pharmaceutical Co., LTD, Production batch number: 81292612811545307188) + lidocaine (Shiyao Silver Lake Pharmaceutical Co., LTD, Production batch number: 83670840065815540594) was injected into the left leg at the dose of 10 mg/2 ml (8 weeks, 1 week/time). The rats were fasted but allowed to drink freely 24 h after the last dose and sacrificed through excessive anesthesia (pentobarbital sodium injection, 150–200 mg/kg).

2. Materials and methods

This study requested informed consent and was approved by the Ethics Committee of Chengdu University of Physical Education (2022.47-48). Randomized controlled animal experiments: A total of 36 2-month-old female SD rats (weighing approximately 200 g and provided with SPF-grade feed) were provided by Chengdu Dasuo Biotechnology Co., LTD (license No.: SCXK-2013-24). The rats were kept at the relative humidity of 55%–70% and room temperature (20 °C–25 °C). After normal feeding for 1 week, the rats were divided into the normal group (n = 10) and the tendinopathy model group (n = 26). The tendinopathy model group was established in reference to Khan et al. [15] as follows: A rat was fixed in the prone position on a small animal surgical operating table then injected with 300 ng of prostaglandin E2 in the middle of its left Achilles tendon once a week for a total of four times. After 4 weeks of prostaglandin E2 injection, two animals were randomly taken from the normal and tendinopathy model groups for HE and Masson staining to observe fiber arrangement, cell density, and inflammation and confirm whether the animal model had been successfully established. Subsequently, the tendinopathy model group was divided into the model group (MG, n = 8), ASA VI group (AG, n = 8), and triamcinolone acetonide + lidocaine group (TG, n = 8).

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2.1. Histological and histochemical examination of samples

The Achilles tendon tissue of the left lower limbs was collected, fixed with formaldehyde, embedded in paraffin, longitudinally sectioned at the thicknesses of 4–6 μm, and subjected to hematoxylin–eosin staining and Masson staining. Tendon fiber morphology, nuclei, vascular proliferation, and inflammatory cell infiltration were observed under a light microscope.

2.2. RT-PCR

RT-PCR was used to determine the mRNA expression levels of the indicators of TSPC differentiation, namely, osteogenic lineage differentiation indicators, the HIF-1α/Hh pathway factor, HIF-1α,
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sonic hedgehog (Shh), patched1 (Pch1), smoothened (Smo), GlI family zinc finger 1 (Gli1), negative regulator of hedgehog signaling (SuFu), cycin-D1 (Cyc-D1), cycin-E1 (Cyc-E1), and cMyc; the indicators of tenogenic differentiation, including sleraxis (Scx), mohawk (Mkx), anti-EYA1 (EYA1), and anti-EYA2 (EYA2); and extracellular matrix synthesis factors, including metallopeptidase 3 (MMP3), tissue inhibitor of MMP3 (TIMP3), collagen I (COL1), collagen alpha-3 (COL3), and tenasin-C (TNC).

RT-PCR was also used to determine angiogenic factor indicators, including vascular endothelial growth factor A (VEGF-A), kinase insert domain receptor (KDR), and von Willebrand factor (VWF), and the inflammatory factor indicators interleukin-1 (IL-1), interleukin-4 (IL-4), and interleukin-10 (IL-10).

2.3. Western blot analysis

After grinding the tendon tissue, the total protein was determined by using a BCA kit, and the expression levels of Gli1, Pch1, and Shh were determined through Western blot analysis. The bands were exposure-scanned with Teneng GIS chassis control software V2.0, and the results were expressed as the relative expression level of the target protein as follows: Relative expression amount of the target protein – target protein integral optical density value (IOD)/internal reference integral optical density value (IOD).

2.4. Immunofluorescence microscopy

Shh, Pch1, Gli1, Scx, and SRY-box transcription factor 9 (Sox9) were also visualized by immunofluorescence staining with endothelial cell-specific markers. The levels of platelet endothelial cell adhesion molecule-1 (PECAM-1)/CD31 and endomucin were also determined.

2.5. Statistical analysis

Data were processed with SPSS17.0 statistical software (SPSS, USA) and GraphPad Prism 8.0.1.244 × 64 (GraphPad, USA). Measurement data are presented as mean ± SD. Multiple comparisons between groups were performed by using one-way ANOVA with Bonferroni adjustment to ensure that the overall type I rate for each ANOVA was not greater than 0.05. The significance level was 0.05.

3. Results

3.1. Pathological observations on the tendinopathy model group

Fig. 1 shows that the fiber tissue arrangement of the rat normal group tendon was highly ordered and intact without an obvious wavy appearance and abnormal cell density. The tendon fibers in the tendinopathy model group were loose and ruptured with a wavy fiber arrangement, incomplete structure, abnormal neo-vascular proliferation, inflammatory cell infiltration, significantly rounded nuclei, and significantly increased cell density, suggesting successful modeling.

3.2. ASA VI inhibits inflammation and improves the microenvironment

Fig. 2 shows that IL-1, IL-4, and IL-10 mRNA expression levels in the MG rats were significantly different from those in the NG rats (P < 0.01); The co-expression of the proinflammatory factor IL-1, the inflammation suppressor IL-4, and IL-10 mRNA in the Achilles tendon tissue of AG rats was significantly different from that in the Achilles tendon tissue of MG rats (P < 0.01); The mRNA expression levels of IL-1, IL-4, and IL-10 in the Achilles tendon tissues of TG rats was significantly different from those in the Achilles tendon tissues of MG rats (P < 0.01).

3.3. Angiogenesis functions in repairing tendinopathy

Fig. 2 shows that that the expression levels of MMP3, TIMP3, VEGF-A, KDR, and VWF in the Achilles tendon tissue of MG rats were significantly different from those in the Achilles tendon tissue of NG rats (P < 0.01); The mRNA expression levels of MMP3, TIMP3, VEGF-A, KDR, and VWF in the Achilles tendon tissue of AG rats were significantly different from those in the Achilles tendon tissues of MG rats (P < 0.01).

The mRNA levels of MMP3, TIMP3, VEGF-A, KDR, and vascular hemophilia factor VWF in the Achilles tendon tissue of TG rats were significantly different from those in the Achilles tendon tissue of MG rats (P < 0.01). The results of immunofluorescence staining in Fig. 2 show that the endothelial cell-specific marker CD31/endomucin level was significantly higher in injured tendon tissue than in normal tendon tissue, suggesting that CD31/endomucin is upregulated in tendinopathy.

3.4. Tendinous differentiation of TSPCs

Fig. 3 illustrates that the mRNA expression levels of Scx, Mkx, EYA1, EYA2, COL1, COL3, and TNC in the Achilles tendon tissues of MG rats were significantly different from those in the Achilles tendon tissue of NG rats (P < 0.01). The mRNA expression levels of Scx, Mkx, EYA1, EYA2, COL1, COL3, and TNC in the Achilles tendon tissues of AG rats were significantly different from those in the Achilles tendon tissue of MG rats (P < 0.01); The mRNA expression levels of Scx, Mkx, EYA1, EYA2, COL1, COL3, and TNC in the Achilles tendon tissues of TG rats were significantly different from those in the Achilles tendon tissue of MG rats (P < 0.01); Through immunofluorescence staining, we found that Sox9 levels were significantly higher and Scx levels were...
significantly lower in injured tendon tissue than in normal tendon tissue (Fig. 3). These results indicate that in tendinopathy, Sox9 is upregulated and Scx is downregulated.

3.5. HIF-1α mediates the Hh pathway in tendinopathy repair by ASA VI

Fig. 4 shows that HIF-1α, Shh, Ptch1, Smo, Gli1, SuFu, Cyc-D1, Cyc-E1, and c-Myc mRNA expression levels in MG rats were significantly different from those in NG rats (P < 0.01). HIF-1α, Shh, Ptch1, Smo, Gli1, SuFu, Cyc-D1, and c-Myc mRNA expression levels in the Achilles tendon tissues of TG rats were significantly different from those in the Achilles tendon tissues of MG rats (P < 0.01); Protein test results (Fig. 4) show that the protein expression levels of Shh, Ptch1, and Gli1 varied significantly in the Achilles tendon tissues of MG rats compared with those in NG rats (P < 0.01). The expression levels of Gli1, Shh, and Ptch1 in the Achilles tendon tissues of AG rats were significantly different from those in the Achilles tendon tissues of MG rats (P < 0.01). The expression levels of Gli1, Shh, and Ptch1 in the Achilles tendon tissues of AG rats were significantly different from those in the Achilles tendon tissues of MG rats (P < 0.01). MMP3, VEGF-A, KDR, and VWF mRNA in Achilles tendon tissues were significantly downregulated after ASA VI treatment. Previous studies have revealed that MMP-3 expression is upregulated in biopsy specimens of degenerative tendons and that MMP-3 synthesis is accompanied by increased neovascularization and the emergence of VEGF [21,22]. VEGF is an important factor involved in angiogenesis [3,23]. Angiogenesis induced by VEGF promotes tissue repair and remodeling in injured tendons. Alternatively [24,25], VEGF stimulates changes in the levels of MMPs in vascular endothelial and smooth muscle cells, and tendon degeneration is associated with MMP-mediated enhanced collagen turnover [26,27]. VWF is a glycoprotein that is

4. Discussion

4.1. ASA VI promotes muscle tendon repair

This study showed that ASA VI injection into rat Achilles tendon tissue significantly decreased IL-1β expression and significantly increased the mRNA levels of the inflammatory suppressors IL-4 and IL-10, indicating that ASA VI intervention improved the microenvironment wherein TSPCs are located and reduced inflammation. Previous studies have demonstrated the presence of degraded collagen fibers; disordered collagen structure arrangement; increased mucus, proteoglycan, and peroxide reductase contents; tendon cartilage-like metaplasia; and increased cell degeneration in injured tendon tissue [16,17]. However, ASA VI has anti-inflammatory and antioxidant effects and inhibits IL-6 and IL-1[18–20]. The results of this study are consistent with the above findings.

This study showed that MMP3, VEGF-A, KDR, and VWF mRNA in Achilles tendon tissues were significantly downregulated after ASA VI treatment. Previous studies have revealed that MMP-3 expression is upregulated in biopsy specimens of degenerative tendons and that MMP-3 synthesis is accompanied by increased neovascularization and the emergence of VEGF [21,22]. VEGF is an important factor involved in angiogenesis [3,23]. Angiogenesis induced by VEGF promotes tissue repair and remodeling in injured tendons. Alternatively [24,25], VEGF stimulates changes in the levels of MMPs in vascular endothelial and smooth muscle cells, and tendon degeneration is associated with MMP-mediated enhanced collagen turnover [26,27]. VWF is a glycoprotein that is
synthesized and released by endothelial cells. It is involved in the processes of coagulation and thrombosis and the expression and synthesis of adhesion factors. It is a sensitive indicator \[28,29\] that specifically reflects the degree of damage to vascular endothelial cells. CD31/endomucin fluorescence was attenuated after ASA VI treatment. CD31 is one of the characteristic markers of vascular endothelial cells and is involved in endothelial cell migration and angiogenesis progression \[30,31\]. Some scholars have classified tendon injury as an angiogenesis-dependent disease. In healthy tendons, the blood vessels are relatively tight, establishing a relatively closed interface that limits the free passage of blood components through the tendon \[32\].

However, inflammation is accompanied by angiogenesis undergoing rest. After the activation period, it enters the remodeling stage, and the proangiogenic mediators in the wound gradually degrade and the expression of antiangiogenic factors increases, leading to the degeneration of the vascular network \[23\]. The results of this study confirm the above views and indicate that ASA VI treatment inhibits excessive vascular proliferation then promotes Achilles tendon repair.

Notably, the present study found that the mRNA levels of the tendonogenesis-related transcription factors Scx, Mkx, EYA1, EYA2, COL1, COL3, TNC were significantly upregulated in Achilles tendon tissues. Meanwhile, immunofluorescence staining showed that Scx was elevated and Sox9 decreased. Studies have found that TSPCs exhibit self-renewal and multidirectional differentiation and participate in tendon tissue homeostasis, repair, and disease occurrence \[5\]. Moreover, previous studies have shown that Scx and Sox9 are involved in the production of TSPCs \[33,34\] and that Mkx is required for tendon differentiation \[35–37\]. The skeletal muscle transcription factors Six1/2 and EYA2 have been reported to play an important role in limb tendon development \[38\]. Scx, COL1, COL3, and TNC are commonly used tendon-associated markers \[39\]. In inflammatory reactions, the altered phenotype of TSPCs, the enhanced ability to differentiate into cartilage, and weakened differentiation into the tendon system are some of the main factors leading to poor tendon repair and the ectopic ossification of TSPCs \[20\]. The presence of serum is required for the differentiation of TSCs \[23\]. However, the excessive supply of blood components after tendon injury may lead to adverse effects, such as faulty cell

Fig. 3. Asperosaponin VI promote TSPCs tenogenesis differentiation in Achilles tendinopathy. N: Normal group; M: Model group; A: ASA VI group; T: Triamcinolone acetonide + lidocaine group. qPCR analyses of Scx, Mkx, EYA1, EYA2, COL1, COL3, TNC mRNA. immunofluorescent staining of Scx, Sox9 (20X). \(* * P < 0.01\).
differentiation, disturbed matrix organization, and concomitant mechanical function impairment [32]. Our results indicate that ASA VI inhibits vasokinogenesis, improves the pathological vascular microenvironment, and subsequently enhances TSPC tendon lineage differentiation and extracellular matrix collagen and tenoprotein synthesis, thus promoting the dual endogenous and exogenous repair of the tendon.

4.2. HIF-1α mediates the mechanism of the Hh pathway in the regulation of TSPC differentiation by ASA VI

The results of this study showed that the injection of ASA VI into rat Achilles tendon tissue prompted the significant downregulation of HIF-1α and a significant decrease in the mRNA levels of the Hh pathway factors Shh, Ptc1, Smo, Gli1, Cyc-D1, Cyc-E1, and c-Myc.
Immunofluorescence staining revealed a decrease in Shh, Ptc1, Gli1, and Sox 9. We believe that ASA VI reduced HIF-1α expression and Shh in an autocrine manner; further reduced Ptc1; enhanced Smo inhibition; reduced Gli protein in the nucleus; and inhibited the transcription of the downstream target genes CyclinD, CyclinE, and Myc, thus inhibiting the Hh pathway in the hypoxic microenvironment and downregulating the level of the osteogenic factor Sox 9. The further inhibition of the abnormal bone lineage differentiation of TSCs suggested that HIF-1α may be an inducer of Hh signaling pathway activation and that HIF-1α activation is associated with promoting the osteochondrogenic differentiation of TSPCs.

Some scholars have reported that the expression of HIF-1α was elevated in the rat ectopic ossification model of Achilles tendon severotemy [40]. HIF-2α was significantly up-regulated in tendinopathy expression microarray analysis and was expressed at low levels in normal tendon tissues [41]. The results of this study agree with the above findings. Hh signaling plays an extremely important regulatory role in abnormal tendon differentiation and cartilage and bone development, as well as in the maintenance of the proliferative and self-renewal potential of stem cells; moreover, the dysregulation of Hh signaling leads to ectopic ossification of the tendon, and the removal of Hh signaling prevents ectopic ossification in tendinopathy [44]. Sufu is the main negative regulator of Hh signaling, and its inactivation leads to severe ectopic activation by the Hh pathway [45]. Therefore, the decrease in Hh signaling is sufficient to attenuate tendinopathy by inhibiting ectodermal ossification [46]. Some part supports the conclusions of this study. We conclude that ASA VI can inhibit the osteoblastic lineage differentiation of TSPCs through the HIF-1α/Hh pathway and promote injury and tendon degeneration repair.

This study initially tested the hypothesis that ASA VI improves the pathological vascular microenvironment and downregulates the HIF-1α/Hh pathway to promote tendon lineage differentiation and tendon repair in TSPCs. However, this study has some limitations. First, the improvement in biomechanical performance was not further analyzed. Second, the intervention was not long enough for validation at the cellular level. In the future, we will further reveal the mechanism of ASA VI in regulating TSPC differentiation and tendon repair mediated by HIF-1α/Hh.

5. Conclusion

This study demonstrated that the downregulation of the HIF-1α/Hh pathway by ASA VI promotes the tendinoid differentiation of TSPCs to repair tendons. The effect of ASA VI on HIF-1α levels may be an effective target for the treatment of tendinopathy.

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