Original Paper

Locally Produced IGF-1 Promotes Hypertrophy of the Ligamentum Flavum via the mTORC1 Signaling Pathway

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Key Words

IGF-1 • Hypertrophy • Ligamentum flavum • mTORC1 • Signaling

Abstract

Background/Aims: Narrowing of the lumbar spinal canal is a condition called lumbar spinal stenosis (LSS) and is a high-morbidity problem in the elderly. LSS is commonly caused by hypertrophy of the ligamentum flavum (HLF). Previous studies showed that fibrosis of the ligamentum flavum (LF) largely contributed to HLF. However, the underlying pathomechanism remains unclear. Insulin-like growth factor-1 (IGF-1) is known to have an intimate relationship with fibrosis in various tissues. Nevertheless, currently, there are few studies regarding IGF-1 in HLF. In this study, we investigated the role of IGF-1 in HLF and its potential molecular mechanism of action. **Methods:** First, the IGF-1, phosphorylation of IGF-1 receptor (pIGF-1R), phosphorylation of AKT (pAKT), phosphorylation of S6(pS6), collagen I and collagen III expression levels were examined via immunohistochemistry and Western blotting in LF tissues from patients with LSS or Non-LSS. Second, primary LF cells were isolated from adults with a normal LF thickness and were cultured with different concentrations of IGF-1 with or without NVP-AEW541/rapamycin. **Results:** The results showed that IGF-1, pIGF-1R, pAKT, pS6, collagen I and collagen III protein expression in the LSS group was significantly higher than that in the Non-LSS group. Meanwhile, pIGF-1R, pAKT, pS6, collagen I and collagen III protein expression was significantly enhanced in LF cells after IGF-1 exposure, which can be notably blocked by NVP-AEW541. In addition, pS6, collagen I and collagen III protein expression was blocked by rapamycin. **Conclusions:** Enhanced IGF-1 promotes the synthesis of collagen I and collagen III via the mTORC1 signaling pathway, which eventually contributes to hypertrophy of the ligamentum flavum.

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Introduction

Lumbar spinal stenosis (LSS) has a high rate of morbidity in elderly patients [1-3]. LSS can lead to severe low back pain, leg pain, intermittent claudication and disability [4, 5]. There are several pathogenic factors that lead to LSS, such as disc degeneration, facet degeneration, and hypertrophy of the ligamentum flavum (HLF)[6]. Among these pathogenic factors, HLF plays a vital role in LSS[7-10]. Previous studies have indicated that fibrosis of the ligamentum flavum stimulated by chronic inflammation is considered to be the most important pathological process of HLF [11, 12]. In the hypertrophied ligamentum flavum, severe fibrosis was observed. Fibrosis was primarily caused by increased collagen I and collagen III[13-15]. Meanwhile, most investigators accept the theory that mechanical stress alteration of the ligamentum flavum stimulates the release of pro-inflammatory cytokines and growth factors, which ultimately induce fibrosis of the ligamentum flavum [13, 16, 17].

Insulin-like growth factor-1 (IGF-1) is an important growth factor in the mammalian tissue microenvironment and plays an important role in tissue fibrosis by activating the mTORC1 signaling pathway [18-20]. In the skeletal system, the release of IGF-1 has been demonstrated to correlate with immediate mechanical stress [21, 22]. Under mechanical loading, IGF-1 is released into the matrix, which induces downstream production of collagen fibers and, ultimately, results in fibrosis and hypertrophy of different tissues [23-27]. However, despite these previous studies on the potential role of IGF-1 in tissue fibrosis and hypertrophy, the interconnection between IGF-1 and HLF has not been thoroughly explored.

In the current work, we hypothesized that IGF-1 may promote the production of collagen fibers that contribute to HLF by activating the mTORC1 signaling pathway. To verify our hypothesis, we first evaluated the protein expression of IGF-1, mTORC1-signaling-pathway-related proteins (pIGF-1R, pAKT and pS6), collagen I and collagen III in LF tissues. Next, to investigate the potential molecular mechanism, we conducted *in vitro* studies on isolated primary LF cells.

Materials and Methods

Ligamentum flavum tissue samples

Ligamentum flavum tissue samples were collected from twenty patients (10 males and 10 females, average age: 59.60 years) who underwent decompressive laminectomy for LSS. Meanwhile, twenty patients (8 males and 12 females, average age: 56.05 years old) without LSS (Non-LSS) who underwent a similar surgery were selected as the control group. The ligamentum flavum tissues were sampled from the L4/5 segments and rinsed in physiological saline 3 times. Part of each sample was fixed in 4% paraformaldehyde for hematoxylin and eosin (H&E) staining, Masson's trichrome staining and immunohistochemical analysis. The remaining samples were stored immediately in liquid nitrogen for subsequent Western blot analysis.

Informed consent was obtained from each patient, and the experiments performed in this study were approved by the Institutional Ethics Review Board of the Third Affiliated Hospital of Southern Medical University, Guangzhou, Guangdong, China.

Measurement of LF thickness

LF thickness was measured by obtaining preoperative magnetic resonance images (MRI, Philips, Amsterdam, Netherlands) from all 40 patients. On axial T2-weighted images through the facet joint [28, 29], the maximum thickness of the LF was measured by an experienced spine surgeon using the Picture Archiving and Communication Systems (PACS) software (The Third Affiliated Hospital of Southern Medical University, Guangzhou, China). Each ligamentum flavum was measured three times, and the average value was taken as the final LF thickness (Fig. 1).

Fibrosis evaluation of LF

LF tissue samples were fixed in 4% paraformaldehyde for 48 h before being processed and were embedded into paraffin blocks. The samples were cut into 4 µm thick sections using a paraffin microtome

(RM2125 RTS, Leica, Wetzlar, Germany). The sections were stained using H&E staining and Masson's trichrome staining kits (JianChen, Nanjing, China). H&E staining showed the morphology and structure of the LF. Simultaneously, the degree of LF fibrosis was evaluated according to the results of Masson's trichrome staining. Three randomly selected fields were observed under 200× magnification of each sample section to determine the grade of fibrosis. Similar to previous research [14], the degree of LF fibrosis was classified into 5 grades: Grade 0, ≤20% of the area of the sample was stained blue; Grade 1, between 21% and 40% of the area of the sample was stained blue; Grade 2, between 41% and 60% of the area of the sample was stained blue; Grade 3, between 61% and 80% of the area of the sample was stained blue; and Grade 4, over 81% of the whole area of the sample was stained blue.

Immunohistochemical analyses

LF tissue sections were deparaffinized and incubated for 10 minutes in 3% H₂O₂ to quench endogenous peroxidase activity. For antigen retrieval, the sample sections in citrate buffer were heated for 16 h at 60°C. Next, the sample sections were washed 3 times for 5 minutes each using phosphate-buffered saline (PBS). Non-specific binding was blocked for 30 minutes with 5% horse serum. Primary antibodies against IGF-1 (1:200, Abcam, Cambridge, UK), pIGF-1R (1:100, Abcam), pAKT (1:100, Abcam), pS6 (1:200, ABclonal, Boston, MA), collagen I (1:100, Abcam) and collagen III (1:100, Abcam) were applied overnight at 4°C. After being washed 3 times for 5 minutes each with PBS, tissue sections were incubated with a goat-anti-rabbit horseradish peroxidase-conjugated secondary antibody (1:100, RayBiotech, Beijing, China) for 1.5 h at room temperature, and the immunostain signal was developed using 3, 3'-diaminobenzidine (DAB, ZSGB-Bio, Beijing, China). The numbers of positive cells (stained brown) were counted by 3 volunteers in a double-blinded manner in 5 randomly selected fields (400×) using the ImagePro 4.5 software (Media Cybernetics, Rockville, MD).

Transmission electron microscopy detection

Ligamentum flavum tissues were cut into blocks of approximately 1.0 mm³ and were fixed in 2.5% glutaraldehyde for 4 h. The samples were dehydrated with propylene oxide and were embedded in Epon. Finally, 100 nm thick ultrathin sections were cut using an ultramicrotome (UC7, Leica, Wetzlar, Germany), stained with 2% uranyl acetate, and observed under a transmission electron microscope (H-7500, Hitachi Technology, Tokyo).

Culture and identification of LF cells

LF samples of normal thickness were aseptically obtained from 8 adult patients who underwent spinal surgery. After washing in physiological saline 3 times, the LF samples were minced into pieces of approximately 0.5 mm² and digested for 1.5 h at 37°C using 0.2% type I collagenase (Sigma-Aldrich, St. Louis, MO). The minced/digested LF tissue samples were washed with Dulbecco's Modified Eagle's medium (DMEM, Gibco, New York, NY) and were centrifuged 3 times (1000r/min, 5 min). Finally, the LF tissue samples were placed in 6-well plates (Corning-Costar, New York, NY) in DMEM supplemented with 10% fetal bovine serum (Gibco), 100 U/ml penicillin and 100 mg/ml streptomycin (Gibco) and incubated in a 5% CO₂ humidified incubator at 37°C. The medium was changed every 3 days. After approximately 10 days, LF cells started to migrate out of the LF tissue samples and formed a monolayer. At 90% confluence, LF cells were passaged 1:3.

The morphology of LF cells was observed under a phase contrast microscope (Axio Scope A1, Zeiss, Oberkochen, Germany). The expression of collagen I and vimentin was detected via immunostaining (see above) and was observed under a confocal microscope (FV1200, Olympus, Tokyo, Japan) to identify the cell type. LF cells of the third passage were treated with different concentrations of recombinant human IGF-1

Fig. 1. Measurement of the ligamentum flavum thickness by MRI. (A) The target area was selected for analysis; (B) the arrow represents the ligamentum flavum thickness at the facet joint level.

(0, 1, 10, 100 ng/ml, Sino Biological, Beijing, China) with or without 100 ng/ml NVP-AEW541 (MedChem Express, Monmouth Junction, NJ) or 10 ng/ml rapamycin (Alexis Biochemicals, Lausen, Switzerland) for 24 h.

Cell viability assays

The viability of LF cells was evaluated using the 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide colorimetric assay (MTT, Solarbio, Beijing, China). LF cells were seeded in 96-well plates at 1×10^4 cells per well and were incubated at 37°C under 5% CO₂ for 24 h. LF cells were washed with PBS 2 times and incubated in 90 µl of DMEM with 10 µl of MTT solution in each well for 4 h. After medium removal, 110 µl of dimethyl sulfoxide (DMSO) was added to each well to dissolve formazan, and the absorbance of the solution was measured at 490 nm using a multi-mode reader (HTX, Gene, Shanghai, China).

Western blotting assays

LF tissues and LF cells were lysed in Laemmli buffer (62.5 mM Tris-HCl, pH 6.8, 2% sodium dodecyl sulfate, 10% glycerol, 50 mM dithiothreitol, and 0.01% bromophenol blue) for 10 minutes at 100 °C. The tissue and cell lysates were separated via 8% or 15% sodium dodecyl sulfate polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes (Millipore, Billerica, MA). The membranes were blocked with 5% non-fat milk diluted in Tris-buffered saline for 2 h at room temperature. Next, the membranes were incubated at 4°C overnight with primary antibodies as follows: collagen I (1:1000, Abcam), collagen III (1:1000, Abcam), IGF-1 (1:1000, Abcam), pIGF-1R (1:1000, Abcam), IGF-1R (1:1000, Abcam), pAKT (1:1000, Abcam), AKT (1:1000, Abcam), pS6 (1:100, ABclonal, Boston, MA), S6 (1:1000, ABclonal), and β-actin (1:1000, ABclonal). Next, the blots were washed and incubated with anti-rabbit or anti-mouse immunoglobulin G peroxidase conjugate (1:3000, RayBiotech, Beijing, China) for 1.5 h at room temperature. The signals were detected using an enhanced chemiluminescence kit (Beyotime, Shanghai, China).

Statistical Analysis

The independent-sample t-test was used to compare the Non-LSS group with the LSS group in terms of age, LF thickness and grade of LF fibrosis (IBM SPSS Statistics 22.0, SPSS, Chicago, IL). One-way ANOVA was used to analyze the MTT assay results, and unpaired Student's t-test was used to determine significant differences in the immunohistochemical staining data (GraphPad Prism 5.01, La Jolla, CA). The results are presented as the mean ± SD for all parameters measured. P<0.05 was considered statistically significant.

Results

Ligamentum flavum thickness and fibrosis of the Non-LSS group and LSS group

The ligamentum flavum thickness of each patient was measured via MRI, and the results showed that the mean ligamentum flavum thickness in the Non-LSS group (2.84 ± 0.47 mm) was significantly lower than that in the LSS group (5.76 ± 0.57 mm) (Table 1). Meanwhile, the degree of ligamentum flavum fibrosis was higher in the LSS group compared to that in the Non-LSS group (Table 1). Histological examinations of H&E (Fig. 2 A, B) and Masson's trichrome (Fig. 2 C, D) stained sections showed a significant loss of elastic fibers and an increase in collagen fibers in the LSS group and a lack of obvious alterations in the Non-LSS group.

Ultrastructure of LF tissues in the Non-LSS group and LSS group

The transmission electron microscopy (TEM) results showed that the LF tissues in the Non-LSS group primarily contained elastic fibers, with very few collagen fibers among them (Fig. 3 A), and that the number of elastic fibers was reduced significantly and number of collagen fibers was markedly increased in

Table 1. General data of patients in two groups. Independent-sample t-test; data are presented as the mean ± SD; P < 0.05 is considered to be significant. LF, ligamentum flavum; LSS, lumbar spinal stenosis

	Non-LSS Group (n=20)	LSS Group (n=20)	P value
Age (years)	56.05 ± 9.24	59.60 ± 7.80	0.197
LF thickness (mm)	2.84 ± 0.47	5.76 ± 0.57	<0.001
Grade of LF fibrosis	0.2 ± 0.41	3.1 ± 0.79	<0.001
Gender	8 males, 12 females	10 males, 10 females	
Lumbar level	L4/5	L4/5	

Fig. 2. Morphology and fibrosis of ligamentum flavum tissues in the Non-LSS group and LSS group. Representative images of H&E staining of the Non-LSS group (A) and LSS group (B), with elastic fibers stained crimson and collagen fibers stained pink; representative images of Masson's trichrome staining of the Non-LSS group (C) and LSS group (D), with elastic fibers stained red and collagen fibers stained blue; representative images of collagen I immunohistochemical staining (brown stain) of the Non-LSS group (E) and LSS group (F); representative images of collagen III immunohistochemical staining (brown stain) of the Non-LSS group (G) and LSS group (H). The scale bar indicates 50 μ m.

Fig. 3. Transmission electron microscopy examination of ligamentum flavum tissues of the Non-LSS group and LSS group. In the Non-LSS group, oval elastic fibers form the majority, with few collagen fibers among them (A); inactive ligamentum flavum cells contain a small amount of cytoplasm and organelles (B). In the LSS group, oval elastic fibers are reduced and collagen fibers are increased in number (C), and active ligamentum flavum cells have an abundant cytoplasm and more organelles (D). EF: elastic fibers; CF: collagen fibers; LFC: ligamentum flavum cell; blue arrows point to the mitochondria, and red arrows point to the rough endoplasmic reticulum. (A) and (C): 8000 $\times$; (B) and (D): 40000 $\times$; the scale bar indicates 5 μ m in (A) and (C) and 1 μ m in (B) and (D).

the LSS group (Fig. 3 C). At 40000 $\times$ magnification, inactive ligamentum flavum cells were found to have a small number of cytoplasm with few mitochondria and a rough endoplasmic reticulum in the Non-LSS group (Fig. 3 B), while active ligamentum flavum cells were found to have an abundant cytoplasm with a large number of mitochondria and a rough endoplasmic reticulum in the LSS group (Fig. 3 D).

High levels of IGF-1, pIGF-1R, pAKT, pS6, collagen I and collagen III in the LSS group

To examine the potential involvement of IGF-1 signaling in LSS, immunohistochemical staining and Western blot analyses were performed to analyze the expression of IGF-1, pIGF-1R, pAKT, pS6, collagen I and collagen III in LF tissues of the two groups. Compared to the Non-LSS group, the number of cells positive for IGF-1 (Fig. 4 A1, A2, A3, A4, A5), pIGF-1R (Fig. 4 B1, B2, B3, B4, B5), pAKT (Fig. 4 C1, C2, C3, C4, C5) and pS6 (Fig. 4 D1, D2, D3, D4, D5) were significantly elevated in the LSS group. Meanwhile, the expression of collagen I (Fig. 2 E, F) and collagen III (Fig. 2 G, H) was obviously higher in the LSS group than that in the Non-LSS group. In addition, as shown by Western blot analyses, remarkable increases were observed in the protein expression levels of IGF-1, pIGF-1R, pAKT, pS6, collagen I and collagen III in the LSS group compared to those in the Non-LSS group (Fig. 5).

IGF-1 stimulated mTORC1 activation, collagen I and collagen III expression in LF cells

In vitro studies with primary LF cells were conducted to investigate the involvement of IGF-1 and the mTORC1 signaling pathway in the HLF process. As shown by immunofluorescence staining (Fig. 6 A, B), most cells were labeled with collagen I or vimentin (markers of LF

Fig. 4. Immunohistochemical staining of IGF-1, pIGF-1R, pAKT and pS6 in ligamentum flavum tissues. Images of immunostaining of IGF-1 in the Non-LSS group (A1, A2) and LSS group (A3, A4); pIGF-1R in the Non-LSS group (B1, B2) and LSS group (B3, B4); pAKT in the Non-LSS group (C1, C2) and LSS group (C3, C4); pS6 in the Non-LSS group (D1, D2) and LSS group (D3, D4). Quantitative analyses of cells positive for IGF-1 (A5), pIGF-1R (B5), pAKT (C5) and pS6 (D5) (arrows indicate positive cells; scale bar=50 μ m; *** indicates $P<0.001$).

Fig. 5. Protein expression levels of IGF-1, pIGF-1R, pAKT, pS6, collagen I and collagen III in ligamentum flavum tissues. Levels of IGF-1, pIGF-1R, pAKT, pS6, collagen I and collagen III are obviously higher in the LSS group than those in the Non-LSS group.

Fig. 6. Identification and viability test of ligamentum flavum cells. Immunofluorescence staining of collagen I (A) and vimentin (B); quantitative analyses of the MTT assay on ligamentum flavum cells cultured with different concentrations of IGF-1, showing no obvious alterations of cell viability (C, $P=0.58$). Immunofluorescence was visualized using FITC (Green), and nuclei were visualized using DAPI (Blue); scale bar = 50 μ m.

cells), suggesting a high purity of LF cells was obtained. In addition, no apparent alteration of cell viability was found in LF cells after being exposed to different concentrations of IGF-1 (Fig. 6 C, $P=0.58$), suggesting that IGF-1 exposure exhibited no toxicity in LF cells.

As shown in Fig. 7 and Fig. 8, the protein expression levels of pIGF-1R, pAKT, pS6, collagen I and collagen III in LF cells increased significantly in a dose-dependent manner after exposure to exogenous IGF-1.

Both NVP-AEW541 and rapamycin can block IGF-1-induced collagen I and collagen III overexpression in LF cells

To confirm the role of mTORC1 signaling in IGF-1-induced collagen I and collagen III expression in LF cells, the IGF-1R specific inhibitor NVP-AEW541 and mTORC1 specific inhibitor rapamycin were included in the exogenous IGF-1 exposure experiments. After treatment with 100 ng/ml NVP-AEW541, IGF-1-induced pIGF-1R, pAKT, pS6, collagen I and collagen III overexpression was significantly attenuated in LF cells (Fig. 7). Similarly, after treatment with 10 ng/ml rapamycin, IGF-1-induced pS6, collagen I and collagen III overexpression was obviously decreased (Fig. 8) in LF cells.

Fig. 7. Protein expression levels of pIGF-1R, pAKT, pS6, collagen I and collagen III in ligamentum flavum cells after exposure to exogenous IGF-1 with or without the IGF-1R inhibitor NVP-AEW541. Expression of pIGF-1R, pAKT, pS6, collagen I and collagen III in ligamentum flavum cells increased significantly and in a dose-dependent manner after exposure to various concentrations of exogenous IGF-1 without NVP-AEW541; IGF-1-induced increases in the expression of pIGF-1R, pAKT, pS6, collagen I and collagen III were attenuated significantly with NVP-AEW541.

Fig. 8. Protein expression levels of pS6, collagen I and collagen III in ligamentum flavum cells after exposure to various concentrations of exogenous IGF-1 with or without rapamycin. Levels of pS6, collagen I and collagen III increased significantly and in a dose-dependent manner after IGF-1 exposure without rapamycin; IGF-1-induced overexpression of pS6, collagen I and collagen III was attenuated significantly with rapamycin.

Discussion

HLF is a major pathogenic factor for LSS. LSS can lead to nerve root compression, low back pain and intermittent claudication and can even develop into paralysis [4-6]. Patients with LSS usually suffer from these symptoms for many years, which cause significant morbidity. Previously, numerous studies have investigated the pathological process of HLF, which ultimately results in LSS; however, the molecular basis of HLF remains largely unknown. The normal ligamentum flavum, anatomically, is composed of 80% elastic fibers and 20% collagen fibers [30]. In HLF, elastic fibers gradually crack, become disordered and reduce in number. On the other hand, collagen fibers are constantly produced and accumulate and contribute to HLF [5]. According to previous reports [13-15], the increased collagen fibers are mainly collagen I and collagen III. As collagen I and collagen III production is believed to strongly correlate with HLF [14, 31, 32], in our study, we measured collagen I and collagen III expression in LF tissues of the Non-LSS group and LSS group. In the current study, the expression of collagen I and collagen III in LF tissues was found to be increased significantly and was associated with the increases in LF tissue thickness and degree of fibrosis in the LSS group compared with the Non-LSS group.

To characterize HLF further, ultrastructural comparisons of LF tissues between the two groups were performed by transmission electron microscopy. Our data suggest that there was increased deposition of collagen fibers in the LSS group than in the Non-LSS group. Furthermore, compared to the Non-LSS group, ligamentum flavum cells in the LSS group were found to have an abundant cytoplasm and more mitochondrion and a rough endoplasmic reticulum. Since protein is synthesized in ribosomes on the rough endoplasmic reticulum and the mitochondria provide energy for this process, from an energy metabolism perspective, ligamentum flavum cells have a higher energy metabolism level in the LSS group than in the Non-LSS group, which can explain the increased production of abundant collagen fibers in the LSS group compared to that in the Non-LSS group.

To explore the potential pathophysiological mechanisms of HLF, many investigators have focused on investigating the relationship between inflammatory factors and HLF. Some

studies suggested that chronic inflammation induced by mechanical stress was a vital impact factor for HLF [11, 12]. It is believed that when suffering from increased mechanical stress, the ligamentum flavum is constantly damaged, causing a local release of inflammation-related factors, such as TNF- α , TGF- β 1, IL-1 and IL-6, which can stimulate LF fibrosis and contribute to the pathological process of HLF [12, 33-36]. Although inflammatory factors are believed to play a crucial role in HLF [5, 11, 37-40], the molecular basis for LF tissue fibrosis remains unclear.

As IGF-1 has previously been found to be a significant growth factor that plays a critical role in the fibrosis process in many tissues [18-20], the current study focused on investigating the potential role of IGF-1 in HLF/LF tissue fibrosis and the molecular mechanism potentially involved. Under mechanical loading, IGF-1 has been previously found to be released by various types of cells, which then induces the production of collagen fibers and, ultimately, results in fibrosis and hypertrophy of different tissues [23-27]. Furthermore, IGF-1 is known to specifically activate mTORC1 signaling and initiate its physiological functions in mammals [18-20, 41-44], and to date, no studies have revealed the potential functions of IGF-1 and mTORC1 signaling in HLF; therefore, the current study hypothesized that IGF-1, through mTORC1 signaling, may contribute to the pathological process of HLF by activating collagen fiber production. Thus, to test our hypothesis, we first performed immunohistochemical analyses of LF tissues, which were significantly increased in LF cells that positively express IGF-1, pIGF-1R, pAKT (the specific upstream marker of mTORC1 signaling) and pS6 (the specific downstream marker of mTORC1 signaling) in the LSS group; these markers were expressed in only a few cells in the Non-LSS group. Furthermore, the Western blot analysis results of LF tissues showed a remarkable increase in the protein expression levels of IGF-1, pIGF-1R, pAKT, pS6, collagen I and collagen III in the LSS group compared to those in the Non-LSS group. These data suggest that a high local expression of IGF-1 in the ligamentum flavum of the LSS group upregulates the phosphorylation of its receptor (IGF-1R) and activates the downstream mTORC1 signaling pathway to enhance collagen I and collagen III production.

To further verify our hypothesis and investigate the potential molecular mechanism involved in the IGF-1 action, we conducted *in vitro* studies on isolated primary LF cells. In 2009, Zhong et al [45]. established a primary culture of human LF cells and distinguished them based on the expression of collagen I and vimentin. Thus, following primary culture, collagen I and vimentin expression was used to identify the purity of the LF cells used in the current study. Our immunofluorescence results showed that the LF cells we isolated exhibited good purity.

Furthermore, while previous studies have reported that both IGF-1 and mTORC1 signaling pathways can stimulate the production of collagen fibers [23-27], it is unknown whether there is a clear interconnection between these two pathways in HLF. Our *in vitro* studies in LF cells showed that exogenous IGF-1 treatment induced a dose-dependent increase of collagen I and collagen III by activating the mTORC1 signaling pathway. Moreover, we found that both NVP-AEW541 (IGF-1R specific inhibitor) and rapamycin (mTORC1 specific inhibitor) blocked IGF-1-induced collagen I and collagen III overexpression in LF cells. These results suggest that the mTORC1 signaling pathway may play a vital mediatory role in IGF-1-induced collagen I and collagen III overexpression in the pathological process of HLF (Fig. 9).

Conclusion

In summary, in this study, we found a high IGF-1 expression level and relevant signaling pathway in HLF. Our study suggested that IGF-1 might contribute to the HLF process by promoting the production of collagen I and collagen III. Moreover, for the HLF process, the mediatory role of the mTORC1 signaling pathway for IGF-1-induced collagen I and collagen III overexpression was also verified. Thus, the current study concluded that locally produced IGF-1 contributed to HLF via the mTORC1 signaling pathway. These findings provide a

Fig. 9. Potential signaling pathway involving mTORC1 activation for IGF-1-induced accumulation of collagen fibers in ligamentum flavum cells. Locally produced IGF-1 binds to the IGF-1 receptor on the membrane of ligamentum flavum cells and further activates the downstream mTORC1 signaling pathway, which promotes the production of collagen I and collagen III and, eventually, contributes to HLF.

novel aspect for understanding the pathogenesis of HLF. Due to the absence of HLF animal models, in the current study, we did not evaluate whether IGF-1 inhibition attenuated the HLF process *in vivo*. Therefore, one important component of our further work is to establish an HLF animal model and to verify IGF-1 action in the HLF process *in vivo*.

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Disclosure Statement

All authors state that they have no conflict of interests.

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