Research Article

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Treatment of adhesions after Achilles tendon injury using focused ultrasound with targeted bFGF plasmid-loaded cationic microbubbles

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Abstract: Nonviral targeting technology has become promising as a form of gene therapy for diseases and injuries, such as Achilles tendon injuries. In this study, we used avidin–biotin bridge and positive–negative charge attractions to load the intercellular adhesion molecule-1 (ICAM-1) antibody and the basic fibroblast growth factor (bFGF) plasmid onto the surface of the microbubbles. The saturated loading capacity for 1.1×10^8 microbubble was $6.55 \pm 0.53 \mu\text{g}$. We established the ICAM-1 antigen microenvironment using tumor necrosis factor- α -stimulated human umbilical vein endothelial cells and found the targeting ability of the prepared microbubbles *in vitro*. *In vivo*, we also found that the injected targeted bFGF gene microbubbles expressed the bFGF gene better when compared with that of the control group. Furthermore, we evaluated adhesions after Achilles tendon injuries in rabbits using hematoxylin and eosin and immunohistochemical (IHC) staining methods. The collagen fibers were properly arranged in the tendon, and there was greater cellularity inside the tendon sheath and a clearer boundary between the internal and external tendon sheath than that of the control group. IHC staining showed

greater ICAM-1 expression inside the tendon sheath when compared with outside the tendon sheath. In conclusion, targeted microbubbles can be a useful carrier of genes to provide gene therapy for the prevention of adhesions after tendon injury.

Keywords: microbubbles, ICAM-1, bFGF, tendon adhesion, gene therapy

1 Introduction

Significant progress has been made in the surgical repair of tendons; adhesions frequently occur during the healing process in Achilles tendon injuries, which seriously affect joint function recovery. Therefore, reducing adhesions during tendon healing with a quick restoration of tendon function has become an intense area of research [1].

Currently, the methods to prevent adhesions include drug therapy, physical barriers, bionic sheath membranes, physical rehabilitation training, and gene therapies. Gene therapy has become an intense area of research because of its ability to be expressed in high quantities and to induce high protein activity while having minimal effects in surrounding tissues. Many researchers have found that cytokines, such as IGF-1 (insulin-like growth factor 1), TGF- β 1 (transforming growth factor-beta 1), and bFGF (basic fibroblast growth factor), have significantly improved Achilles tendon injury repair in animal models. bFGF is a heparin-binding growth factor, which is a peptide composed of 146 amino acids. Exogenous bFGF accelerated wound healing in various injuries [2]. Nakanishi et al. found that the tendon mechanical strength significantly improved in the group that received a composite bFGF coating in the suture line when compared with the control group in a rabbit model of synovial tendon repair [3–5]. Since exogenous healing is the primary cause of tendon adhesions, the question now is how to direct bFGF to promote endogenous healing within the

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tendon sheath, while avoid it works outside the tendon sheath.

Intercellular adhesion molecule-1 (ICAM-1) is a member of the immunoglobulin family, which is mainly expressed on activated endothelial cells and other antigen transmitter cells, mediates the adhesion reaction between endothelial cells and leukocytes, and is almost not expressed in normal tissues. Research showed that the expression of ICAM-1 on the surface of Achilles tendon cells increased significantly in the model of Achilles tendon inflammation. Therefore, in this study, the Achilles tendon cells expressing ICAM-1 surface antigen after injury were used as target receptors to prepare targeted ultrasound microbubbles carrying the ICAM-1 antibody.

Acoustic microbubbles could be used as a gene carrier to treat diseases [6]. Targeted ultrasound microbubble destruction technology uses microbubble contrast agents as carriers to make an adherent surface or to encapsulate target genes. Targeted acoustic microbubbles containing target genes are injected intravenously to reach the target tissues, where the bubbles are broken to release the gene using ultrasonic irradiation.

In this study, we prepared targeted acoustic microbubbles carrying the bFGF gene and detected the gene carrying efficiency and targeting ability of this conjugated substance *in vitro* and *in vivo*. The rabbit model of Achilles tendon injuries has postoperative inflammation that is established by local incision and suture. We used this model to explore the efficiency by which the prepared microbubbles could prevent postoperative adhesions after Achilles tendon injury.

2 Materials and methods

2.1 Preparation of the targeted microbubble contrast agent with the bFGF gene

1,2-Distearoyl-*sn*-glycero-3-phosphocholine (DSPC; 561.9 μ L; Avanti Polar Lipids Inc., USA), 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine-*N*-[biotinyl(polyethylene glycol) 2000] (DSPE-PEG 2000-Biotin; 93.5 μ L; Avanti Polar Lipids Inc., USA), and polyethyleneimine-600 (94.7 μ L; Avanti Polar Lipids Inc., USA) were used to prepare biotinylated cationic microbubbles (BCMs). The rabbit ICAM-1 primary antibody (20 μ L; Biosynthesis Biotechnology Co, Ltd, Beijing, China), the bFGF plasmid (bFGFp; 7 μ L; ACRO Biosystems, Beijing, China), and the prepared BCMs (500 μ L) were mixed using a biotin-avidin bridge to prepare the targeted bFGF gene microbubbles. Unbound antibodies and bFGFp were removed by washing

three times with phosphate-buffered saline (PBS; Hua Maik Biotechnology Co, Ltd, Beijing, China).

The average particle size and the concentration of the prepared microbubbles were detected using an automatic dilution particle calculator (Particle Sizing System, California, USA), and the charge of the prepared microbubbles was detected using the Zeta potential analyzer (Brookhaven, MA, USA).

To confirm that the prepared microbubbles and ICAM-1 antibody were conjugated, three groups were extracted from the ICAM-1 targeted biotinylated microbubbles, the ICAM-1 targeted biotinylated microbubbles with the bFGF gene, and the biotinylated control microbubbles. Dylight 488 goat anti-rabbit IgG (H + L) (1 mL; 1:100 dilution; BOSTER Biological Technology, Ltd, Wuhan, China) was added to each group. The fluorescence distribution was observed in each of three groups using an inverted fluorescence microscope (Olympus Corporation). The experiments were performed three times.

2.2 Ultrasound imaging of targeted biotinylated cationic microbubbles (TBCMs) carrying the bFGF gene in rat livers

Twelve Sprague-Dawley rats weighing 200 ± 20 g (Medical Laboratory Animal Center, Guangdong, China) were divided into four groups and intraperitoneally injected with 0.2 mL of BCMs, TBCMs, TBCMs carrying bFGF, and SonoVue microbubbles, respectively, then followed by an injection of 0.5 mL of normal saline. The rat livers were imaged with the two-dimensional mode (Mylab90 Esaote, Indianapolis, IN, USA). The ultrasound imaging mode was adjusted (Mylab90, LA523 line probe, frequency: 7–10 MHz, MI: 0.8) until enhanced images of rat liver were achieved.

2.3 Targeting ability of targeted microbubbles carrying the ICAM-1 antibody *in vitro*

Umbilical vein endothelial cells were cultured in 12-well culture plates to a confluency of 70–80% and divided into control group (normal medium), targeted microbubbles group (microbubbles carrying the ICAM-1 antibody), tumor necrosis factor alpha (TNF- α) group, and TNF- α targeted microbubbles group (TNF- α microbubbles group). After washing, 5 μ L of 1 ng/ μ L TNF- α liquid (ACRO Biosystems Beijing, China) was added to the TNF- α group and the TNF- α microbubble group. About 5 μ L of

normal medium was added to the control group and targeted microbubble group. The cells were incubated for 6 h at 37°C in 5% CO₂. After washing, cells of the control group and TNF- α group were treated with 2 mL of 4% paraformaldehyde for 15 min, 0.5% Triton X-100 for 20 min at 37°C, and normal goat serum for 2 h. After washing with PBS, cells were incubated with the primary antibody ICAM-1 (1:400; MXB Biotechnologies, Fuzhou, China) at 4°C for 12 h, then stained with secondary antibody (1:400; MXB Biotechnologies, Fuzhou, China) at 37°C for 1 h in dark and finally treated with 4',6-diamidino-2-phenylindole for 15 min in dark. The cells of targeted microbubble group and TNF- α microbubble group were washed three times. Then, the cells of all groups were observed by an inverted fluorescence microscope.

2.4 Rabbit model of Achilles tendon injury and postoperative inflammation

Twelve healthy male New Zealand rabbits weighing 2.0–3.0 kg (Medical Laboratory Animal Center, Guangdong, China) were included for animal model. All procedures in the animal experiments were conducted in accordance with the guidelines developed by the National Institutes of Health and approved by the Institutional Animal Care and Use Committee of Peking University Shenzhen Hospital (March 31, 2014, Permit No. 14-105). Rabbits were anesthetized by phenobarbital sodium injections into the ear vein (1:1 diluted; 0.3 mL/kg; Shanghai New Asia Pharmaceutical Co. Ltd, Shanghai, China). The rabbits were then fixed on the operating table (in the prone position), and the bilateral tendons were fully exposed and disinfected with an iodine solution. The skin of the Achilles tendon was cut into 2 cm longitudinal sections, and the subcutaneous tissue was incised layer by layer until the Achilles tendon was exposed. Then, the tendon was partially cut with transverse sections. The ruptured tendon was fixed with intermittent sutures, sterile saline was used to wash the wound, and the skin was sutured and bandaged with sterile gauze. Penicillin (North China Pharmaceutical Company Limited, Hebei, China) was injected intramuscularly at a dose of 4,00,000 units for the first 3 days post-surgery. The Achilles tendons of the rabbits were then used for the targeted microbubble studies.

2.5 Targeting ability and efficiency of microbubbles [7]

Twelve New Zealand rabbits were randomly divided into four groups: Group 1 was the control group that received

normal saline; Group 2 was the bFGFp group that received the plasmid with bFGF gene; Group 3 was the targeted bFGFp microbubble group that received the contrast agent with the bFGFp and the ICAM-1 to help locate the target; and Group 4 was the bFGFp microbubble group that received the contrast agent with the bFGFp. Each group was injected with 1 mL of the respective fluid into the ear vein. We observed the condition of Achilles' tendons by ultrasonography, and after 20 min, enhancement of the tendon was achieved. Observations and recordings were made after 7 days and 2 weeks, after which time all the rabbits were sacrificed and sections of the Achilles tendons from each group were taken and prepared for histopathology.

2.6 Hematoxylin and eosin (H&E) and immunohistochemical (IHC) staining

The Achilles tendon was fixed with 4% paraformaldehyde and then dehydrated with graded ethanol, dipped in wax, embedded in paraffin, and sectioned. Then, the paraffin sections were dewaxed, colored, rehydrated, transparent, and finally stained by H&E. For IHC, paraffin sections were incubated with the rabbit ICAM-1 primary antibody (Biosynthesis Biotechnology Co, Ltd, Beijing, China) and then with biotin-labeled secondary goat anti-rabbit IgG (BOSTER Biological Technology, Ltd, Wuhan, China). The sections were photographed by using an inverted microscope (Olympus, Germany).

2.7 Western blots

The bFGF protein expression was determined by western blot analysis. The proteins were separated via sodium dodecyl sulfate–polyacrylamide gel electrophoresis in 12% (w/v) polyacrylamide gels and electrotransferred to Millipore polyvinylidene difluoride membranes (Millipore, Bedford, MA, USA). The membranes were blocked in Tris-buffered saline containing 0.05% Tween-20 and 5% nonfat dry milk for 60 min at room temperature. Then, the blots were incubated with a 1:500 dilution of rabbit bFGF primary antibody (Biosynthesis Biotechnology Co., Ltd, Beijing, China) at room temperature in a blocking solution. The membranes were washed in Tris-buffered saline and incubated with biotin-labeled secondary goat anti-rabbit IgG antibody (1:400; BOSTER Biological Technology, Ltd, Wuhan, China) in a blocking solution for 1.5 h. The blots were examined using a chemiluminescent substrate (Santa Cruz Biotechnology, Santa Cruz, CA, USA) according to the

manufacturer's instructions. The protein bands were normalized to glyceraldehyde 3-phosphate dehydrogenase, and the signal intensity of all the blots was quantified by Image J 1.43 software.

2.8 Statistical analysis

SPSS19.0 software was used to analyze the data, and the mean number of the groups was compared with the paired samples *t*-test analyses. A *p*-value of less than 0.05 was defined statistically significant.

3 Results

3.1 Preparation of cationic microbubbles

As shown in Figure 1, SPEI, DSPC, and DSPE-PEG 2000-Biotin were self-assembled to form BCM under a C3F8 environment. The biotin tag incorporated in the microbubble shell can further combine with ICAM-1-biotin antibodies in the suspension via the existence of avidin by biotin-avidin interaction, leading to the TBCMs. The SPEI could enhance the DNA-loading capability of the TBCM.

3.2 DNA loading efficiency of bFGFp onto normal microbubbles and cationic microbubbles

As shown in Figure 2, when the concentration of targeted biotinylated cation microbubbles is within a specific range, the amount of gene bound to the microbubbles is directly proportional to the DNA concentration in the solution. Finally, when the DNA concentration was 17.57 ng/ μ L, the saturated loading capacity for 1.1×10^8 microbubble was $6.55 \pm 0.53 \mu$ g.

3.3 Physical properties of acoustic lipid-encapsulated microbubble contrast agents

The peak value of the microbubble point measured by the Zeta potential potentiometer was 0–100 mV, which is located in the positive charge range (Figure 3a).

Particle sizes and concentrations were measured using the automatic dilution particle counter. BCMs were

with an average particle size of $0.89 \pm 0.11 \mu$ m and an average concentration of $2.63 \pm 0.40 \times 10^9$ /mL (Figure 3b–d). TBCMs were with an average particle size of $0.96 \pm 0.03 \mu$ m and an average concentration of $1.52 \pm 0.18 \times 10^9$ /mL (Figure 3b, c and e). TBCMs carrying the bFGF DNA with an average particle size of $1.08 \pm 0.06 \mu$ m and an average concentration of $1.12 \pm 0.11 \times 10^8$ /mL (Figure 3b, c and f).

3.4 Qualitative ICAM-1 immunofluorescence detection on the surface of cationic microbubbles

ICAM-1 immunofluorescence can be seen as expressed on the surface of the TBCMs and the TBCMs carrying the bFGF gene with the Dylight488 marker. The surface of the normal biotinylated cationic acoustic microbubbles does not express the green fluorescence (Figure 4).

3.5 Ultrasound imaging of the acoustic microbubble contrast agents in rat livers

Hepatic boundaries of the rats were clearly defined using the two-dimensional ultrasound. BCMs, TBCMs, TBCMs carrying the bFGFp, and SonoVue microbubbles were injected into the ear veins of the rats. After 3 s, ultrasonic imaging of the hepatic vascular beds was started. Hepatic parenchyma began to show enhancement after about 15 s, and the enhancement was sustained for approximately 12 min (Figure 5).

3.6 *In vitro* target binding experiments

To detect the targeting ability of targeted microbubbles carrying the ICAM-1 antibody *in vitro*, the expression of ICAM-1 on the cell surface was stimulated by TNF- α . However, when the endothelial cells were not stimulated with TNF- α , the nuclei fluoresced blue, but the cell membranes did not fluoresce green. The green fluorescence in cells stimulated with TNF- α was accompanied by numerous targeted microbubbles binding with ICAM-1 antibody on the surface of the cells. The endothelial cells that were not stimulated with TNF- α had fewer targeted microbubbles in the

Figure 1: Schematic illustration of cationic microbubbles. SPEI600, DSPC, and DSPE-PEG 2000-Biotin first self-assembled to form BCM under a C_3F_8 environment. Then, biotinylated ICAM-1 assembled onto the shell of the TBCM by avidin-biotin interaction. Meanwhile, the plasmid bFGF can further assemble on the TBCM through electrostatic interaction and change to TBCM-bFGF.

Figure 2: The amount of bFGFp bound onto microbubbles. The relationship between the binding rate and bFGF DNA concentration.

that received injections of the bFGFp, moderate limping was noticed, and the wounds were mostly healed, but visible scars were present. Severe adhesions between the Achilles tendon and the surrounding tissues were present, which could not be separated from the surrounding tissues, and more scar tissues were present. In Group 3 that received targeted microbubble with bFGFp and ICAM-1 antibody, rabbits appeared normal. Mild edema was present in the surrounding tissues, but the wound healed well. Smooth Achilles tendons were seen with no obvious adhesions with the surrounding tissues. Mild swelling around the tendon was appeared, and they had good textures and elasticity. In Group 4 that received microbubbles with bFGFp, slight limping was noted with mild edema in the surrounding tissues, and the wounds were almost healed. Smooth Achilles tendons were also seen in this group with mild adhesions in the surrounding tissues. Tendon textures and elasticities were also firm compared with those of Group 3 (Figure 7).

field of view, and most were dispersed in the cells (Figure 6).

3.7 New Zealand rabbit recovery assessments after the Achilles tendon surgery and treatment

In Group 1 that received normal saline, obvious limping was observed; stitches were present in the surgical incision with swelling in the surrounding tissue. Moderate-severe adhesion was found between the Achilles tendon and the surrounding tissues, which was difficult to separate from the surrounding tissues. A few gaps were also seen in the Achilles tendon. In Group 2

3.8 *In vivo* target binding experiments

Using contrast-enhanced ultrasonography (CEUS), we observed the following results. Both Groups A and B had partial incisions of the Achilles tendon. However, Group A received TBCMs, whereas Group B, the control group, received non-TBCMs. Group A had significantly more enhancement on ultrasound than Group B. Group C, which received TBCMs similar to Group A, showed significant enhancement on ultrasound when compared with the control group (Group D), which did not receive incision of the Achilles tendon and received TBCMs (Figure 8).

Figure 3: The concentrations and particle sizes of three kinds of microbubbles: (a) the surface charges of the cationic acoustic microbubbles; (b) the diameter of three kinds of microbubbles; (c) the concentrations of three kinds of microbubbles; (d) particle size distribution of BCMs; (e) particle size distribution of TBCMs; and (f) particle size distribution of TBCMs with the bFGF gene (TBCM-bFGF).

Figure 4: ICAM-expressing microbubbles observed using an inverted fluorescence microscope (a, c and e: white light; b, d and f: fluorescence). a and b: TBCM; c and d: TBCMs with the bFGF gene; and e and f: BCMs.

3.9 H&E staining of the Achilles tendons in rabbits

In experimental Group 3, the collagenous fibers were normally arranged, and the cells were mostly inside the tendons than in the surrounding tissues. The loose connective tissues outside the tendon sheath are not seen within the tendon sheath, and the inner and outer boundaries of the tendon sheath were clear. In the control group (Groups 1 and 2), collagenous fiber had a more disordered arrangement, loose connective tissue was found inside the tendons, and the internal and external boundaries of the tendon sheath were not clear. In addition, the composition of cells inside and outside the tendon sheath was not different. However, in Group 2, more cells were outside the tendon than inside. The control group (Group 4) had normally arranged collagen fibers, and the cells were mostly inside the tendon than outside, but fewer cells inside the tendon were present in Group 4 than in Group 3. The internal and external boundaries of the tendon sheaths were clear (Figure 9).

Figure 5: Hepatic ultrasonography in rats: (a) BCs, (b) TBCs, (c) TBCs carrying the bFGFp, and (d) SonoVue microbubbles.

Figure 6: Photomicrographs from an inverted fluorescence microscope (200 $\times$). (a and b) Cells stimulated with TNF- α in white light and fluorescence model. (c and d) Cells stimulated with TNF- α and combined with targeted ultrasound microbubbles in white light and fluorescence model. (e and f) Cells without TNF- α stimulation in white and fluorescence model. (g and h) Cells without TNF- α stimulation and combined with targeted ultrasound microbubbles in white and fluorescence model.

Figure 7: Adhesions associated with the Achilles tendon in rabbits: (a) Group 1 received normal saline; (b) Group 2 received injections of the bFGFp; (c) Group 3 received targeted microbubble (the contrast agent with bFGFp and the target location factor, ICAM-1 antibody); and (d) Group 4 received microbubbles with bFGFp.

Figure 9: Photomicrographs of H&E staining of the Achilles tendons in rabbits and microscopic observations (100×). (a) Group 1: the control group that received only normal saline; (b) Group 2: the group that received the bFGFp (the plasmid with bFGF gene); (c) Group 3 that received targeted microbubble (the contrast agent with bFGFp and the target location factor, ICAM-1 antibody); (d) Group 4: the group that received the microbubbles with the bFGFp. (Black arrows indicate the external tendon sheath. White arrows indicate the internal tendon sheath).

Figure 8: Contrast-enhanced ultrasound imaging of the Achilles tendons in rabbits: (a) experimental group (partial incision of the Achilles tendon and TBCMs), (b) control group (partial incision of the Achilles tendon and TBCMs), (c) experimental group (partial incision of the Achilles tendon and TBCMs), (d) control group (partial incision of the Achilles tendon and TBCMs).

3.10 IHC staining of rabbit Achilles tendons

In the experimental group (Group 3), ICAM-1 expression inside the tendon sheath is significantly greater than outside the tendon sheath. In the control groups (Groups

1, 2, and 4), ICAM-1 expression inside the tendon sheath was not significantly different from outside the tendon sheath (Figure 10).

3.11 Achilles tendon protein imprinting analysis

Western blot test results show that bFGF protein bands could be observed in all of the groups, and the bFGF protein expression was the highest in the experimental group (Group 3) when compared with the control groups (Groups 1, 2, and 4; Figure 11).

4 Discussion

The acoustic microbubble contrast agent is a kind of microbubble that has a diameter from 1 to 8 μm and contains gas and an outer membrane layer. With intravenous injections, acoustic microbubble contrast

Figure 10: ICAM-1 IHC staining of the Achilles tendons in rabbits and microscopic observation (200×). (a) Group 1: the control group that received only normal saline; (b) Group 2: the group that received the bFGFp (the plasmid with bFGF gene); (c) Group 3: the group that received targeted microbubble (the contrast agent with bFGFp and the target location factor, ICAM-1 antibody); (d) Group 4: the group that received the microbubbles with the bFGFp. (Black arrows indicate the external tendon sheath. White arrows indicate the internal tendon sheath.)

agents can reach the organs or tissues through the pulmonary circulation, which can be seen on ultrasound imaging. Thus, clinicians can image specific areas of interest and thereby increase the ability to diagnosis diseases. However, acoustic microbubbles do not have a special affinity for diseased tissues and do not reside long in tissues [8]. Therefore, researchers have begun to

study targeted acoustic microbubbles which are refer to the microbubbles carrying a specific ligand (such as peptides and antibodies). Targeted acoustic microbubble contrast agents can combine with receptors of organs and tissues through the circulatory system to enhance the ultrasonic imaging times and effects on local target tissues, which improve the specificity imaging of local target tissues and the sensitivity of early diagnosis. Because of these advantages, targeted microbubble contrast agents have become a hot topic in the field of ultrasonic research.

The preparation of the targeted microbubble contrast agents requires the necessary conditions [9]. In this study, the targeted acoustic microbubbles with the ICAM-1 antibody were prepared by using the “biotin–avidin” bridge method, which was considered having high sensitivity, stability, and affinity. Finally, the successful preparation of the targeted microbubble contrast agent with the bFGFp and the ICAM-1 antibody to prevent postoperative adhesions in Achilles tendon injuries becomes a possible choice.

Polyethyleneimine (PEI) is also known as a heterocyclic nitrogen propane and is a highly charged and dense cationic polymer that can be combined with DNA to form nanoscale particles [10]. As a cationic polymer, it protects DNA from enzymatic degradation. For instance, the PEI/DNA complexes escaped lysosomal compartments and degradation after endocytosis [11]. Compared with other cationic polymers, such as polylysine, complete dendritic polymers, and fracture dendritic polymers, PEI has a better acid–base buffering capacity [12,13]. In this experiment, we used PEG-modified phospholipids with PEI, which ensured that the surface of prepared microbubbles was positively charged. Then, bFGF gene was added into the cationic acoustic microbubbles via the mechanical

Figure 11: bFGF protein expression in the Achilles tendon of rabbits. Group 1: the control group that received only normal saline; Group 2: the group that received the bFGFp; Group 3: the group that received the microbubbles with the bFGFp. Group 4: the group that received targeted microbubble (the contrast agent with bFGFp and the target location factor, ICAM-1 antibody).

oscillation method for enhancing transfections. Finally, we succeeded in creating biotinylated cationic liposome-encapsulated acoustic microbubbles that were uniform in size, and the ultrasound images of rat livers showed similar enhancing effects compared with those using the SonoVue microbubbles. Reverse transcription polymerase chain reaction showed a more than 30% greater plasmid carrying capacity of the microbubbles.

TNF- α is a pro-inflammatory cytokine produced by the activated macrophages and is involved in the mediation of early inflammation. During inflammation, endothelial cells are damaged by inflammatory cytokines such as interleukin-1 β (IL-1 β) and TNF- α , which can cause vascular endothelial cell dysfunction and induce expression of several cell growth factors, such as ICAM-1 and vascular cell adhesion molecule-1 [14]. Research has shown that TNF- α can induce ICAM-1 antigen expression on the surface of umbilical vein endothelial cells causing vascular endothelial dysfunction. Moreover, studies have shown that ICAM-1 is not expressed on the surface of normal endothelial cells [15]. Therefore, ICAM-1 can be used to target genes to inflammatory sites for ultrasound imaging studies. In this experiment, we stimulated human umbilical vein endothelial cells with TNF- α and found that the endothelial cell surfaces fluoresced green after stimulation, which was not seen in the endothelial cells that were not stimulated with TNF- α . These results suggested that TNF- α was stimulating ICAM-1 expression on the surface of the endothelial cells. We then incubated the umbilical vein endothelial cells expressing ICAM-1 with the microbubbles carrying the ICAM-1 antibody and found that the fluorescence of the targeted microbubbles surrounding the cells was greater than that of the non-targeted microbubbles using fluorescence microscopy. These results indicated that the targeted ability of the interaction of ICAM-1 of the endothelial cells with the ICAM-1 antibody on the targeted microbubbles *in vitro*.

In this rabbit Achilles tendon study, we demonstrated that the group that received targeted microbubbles containing ICAM-1 antibody and partial incision was the only group to show significant enhancement, using CEUS, as opposed to the group that received microbubbles without ICAM-1 or non-partial incision, which did not show enhancement. This study indicated that the targeted ultrasound microbubbles work *in vivo* and that targeted ultrasound could promote the bFGF gene expression in tendon injuries.

Tendon injuries are common in clinical surgery clinics and can be caused by external causes, such as direct trauma (car accidents), impacts from landing on feet from great heights, strenuous physical activity,

iatrogenic factors, systemic diseases, and chronic degenerative changes [16]. Currently, two main treatments for tendon injuries are nonsurgical and surgical methods. Non-surgical methods include physical therapy, injections, traction therapy, systemic drugs, and adjuvant therapies [17]. Surgical treatments are important in Achilles tendon injuries, and although surgical methods continue to improve, fiber adhesions from the damaged tendons and surrounding tissues remain an obstacle to the functional recovery of patients with Achilles tendon injury.

The tendon healing process includes exogenous healing and endogenous healing [18]. Endogenous healing involves tendon cell proliferation in the tendon sheath and collagen production by tendon fibroblasts to form normal tendon collagen fibers. However, due to the distance between the tendon sheath and surrounding tissues, adhesions with the surrounding tissues would not likely occur. Exogenous healing is caused by angiogenesis and fibrous hyperplasia outside the tendon sheath, allowing both sides of the tendon to be healed. However, granulation tissue can interfere with healing as it promotes adhesion between the tendon and surrounding tissues. Therefore, therapies that support endogenous healing and reduce the exogenous healing are needed. Studies have shown that the growth factors associated with tendon healing are bFGF, epithelial growth factor (EGF), platelet-derived growth factor (PDGF), IGF-1, TGF- β , and vascular endothelial growth factor (VEGF). These growth factors might have specific regulatory functions in the healing of tendon injuries.

bFGF is a heparin-binding growth factor and polypeptide composed of 146 amino acids [19]. The main biological effects of bFGF are as follows: (1) angiogenesis, (2) fibroblast proliferation for wound healing and tissue repair, (3) regeneration of tissues, and (4) nerve regeneration [20]. Alkaline phosphatase can significantly improve type I collagen fiber expression and promote DNA synthesis of tendon fibroblasts [21]. bFGF has been shown to encourage endogenous healing of tendons by promoting collagen secretion and of tenocyte proliferation [22]. It has also been shown to have a dose correlation with tenocyte growth [23,24]. Wang et al. placed bFGF composite membranes in the joints of rat with tendon injuries and found that bFGF promoted endogenous healing of the tendon that was faster than that of the surrounding tissues [25]. This study suggested that bFGF not only accelerates tendon injury healing but also prevents tendon adhesions [26]. However, other studies show that bFGF promotes type III collagen fiber formation and angiogenesis outside the

tendons, causing increased external healing of the tendons [26]. Therefore, we wanted to use bFGF to promote endogenous healing of tendons and reduce exogenous healing to prevent adhesions. To perform these experiments, we used the ICAM-1 antibody with targeted ultrasound microbubbles as the bFGF DNA vector to mediate transfections and showed that the tendon healing and adhesions of rabbits with Achilles tendon injuries were significantly better in the experimental groups when compared with the control groups. H&E and IHC staining showed that the cellularity inside the tendon sheaths was significantly greater in the experimental group when compared with that of the control group, suggesting that endogenous healing was faster than exogenous healing in the tendons of the experimental group. Preliminary conclusions of these experiments are that the targeted ultrasound microbubbles can significantly improve bFGF transfection rates and thereby promote endogenous healing of injured Achilles tendons and can effectively prevent adhesions caused by exogenous healing.

5 Conclusion

In this study, we prepared BCMs with PEI and used the ICAM-1 antibody to localize the transfection to a specific target. The bFGF target gene was attached to the targeted ultrasound microbubble surfaces via the charge effect. TNF- α stimulation induced the ICAM-1 surface expression on human endothelial cells. The results show that the targeted biotinylated microbubbles successfully target endothelial cell surfaces *in vivo* and *in vitro*. Thus, the targeted biotinylated microbubbles with bFGF and ICAM-1 could prevent adhesions after the tendon injury and provide a new pathway to study treatments to prevent adhesion after tendon injuries.

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