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Original article

The influence of connective tissue growth factor on rabbit ligament injury repair

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ABSTRACT

Objectives: The injured anterior cruciate ligament (ACL) is deemed to exhibit an impaired healing response and attempts at surgical repair have not been successful. Connective tissue growth factor (CTGF) is reported to be associated with wound healing, probably through transforming growth factor beta1 (TGF-β1).
Methods: A rabbit ACL injury model was used to study the effect of CTGF on ligament recovery. Quantitative real-time PCR was performed for detection of changes in RNA levels of TGF-β1, type 1 collagen (COL-I), type 2 collagen (COL-II), SRY-related high mobility group-box gene9 (Sox9), metalloproteinase-1 (TIMP-1) as well as matrix metallopeptidase 13 (MMP-13). And expression of related proteins was detected by western blotting.
Results: The current study showed that CTGF could promote the recovery of inured anterior cruciate ligament. It can up-regulate the mRNA and expression of TGF-β1, COL-I, COL-II, Sox9, as well as the tissue inhibitor of TIMP-1, and down-regulated the mRNA and expression of MMP-13, suggesting the curative effect of CTGF on injured rabbit ligament is through regulating these cellular factors.
Conclusion: This finding revealed the mechanism of CTFG's healing role in injured tissues and provided new possibilities of treating injured tissues and wound healing by using CTFG.

1. Introduction

The anterior cruciate ligament (ACL) has long been seen as the primary passive restraint to anterior translation of the tibia with respect to the femur among the contributors to knee joint stability (Kiapour and Murray, 2014; Muhammad et al., 2017). Besides, ACL contributes to knee rotational stability in both frontal and transverse planes because of its specific orientation (Levine et al., 2013; Quatman et al., 2014). It has been the focus of numerous biomechanical/anatomical researches and is one of the most frequently studied structures of the human musculoskeletal system during the past decades. ACL injuries are one of the most common and devastating knee injuries mainly sustained as a result of sports participation (Luo et al., 2016; Hewett et al., 2013).

It was known that ACL had poor healing capacity, with a substantially high rate of failure (40–100%), even after surgical repair using suture (Strand et al., 2005; Taylor et al., 2015). The reconstruction of ACL has remained the gold standard of care for ACL injuries, especially for young individuals and some athletes who aim to return to high-level sporting activities (Liu et al., 2015a; Musahl et al., 2011). However, for now though, surgical treatment of ACL injury is costly, with variable outcomes (Wang et al., 2015; Kiapour et al., 2014), which are often not successful at returning patients to their pre-injury activity level (Ardern et al., 2011; Zaheer et al., 2017). New treatment methods for ACL injury are to be explored, aiming at higher efficiency and lower cost. One potential is using connective tissue growth factor (CTGF), which has been shown to play important roles in lots of biological processes, such as cell adhesion, migration, proliferation, angiogenesis, skeletal development, and tissue wound repair, and is also critically involved in fibrotic disease and several forms of cancers (Jun and Lau, 2011; Hall-Glenn and Lyons, 2011).

CTFG, also known as CCN2 (Jun and Lau, 2011; Hall-Glenn and Lyons, 2011), is a matricellular protein of the CCN family of

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extracellular matrix-associated heparin-binding proteins (Holbourn et al., 2008; Leask and Abraham, 2006). CTFG is known to act in cell adhesion, migration, proliferation, angiogenesis, vascular differentiation and myofibroblast formation, all of which can lead to tissue remodeling and changes in organ structure (Lipson et al., 2012). It was also reported that CTGF was associated with wound healing and virtually all fibrotic pathology (Leask and Abraham, 2006; Brigstock, 2010). Recently, people found that TGF-β1 associated with the increased expression of CTGF, induce the hypertrophy of the LF through the p38 MAPK pathway (Safi et al., 2015; Cao et al., 2016; Nawaz et al., 2017; Samad et al., 2017). CTFG has also been reported to regulate TGF-β1 in in the TGF-β1-induced invasion and migration of hepatocellular carcinoma (Liu et al., 2015b). So it is reasonable to consider the possibility that CTFG is also associated with TGF-β1 in ACL healing.

To investigate the healing effect of CTFG on ACL injury and also to elucidate the mechanism of this effect, we constructed an ACL injury model with rabbits and looked into some TGF-β1 associated cellular factors involved in tissue regeneration and wound recovery. It showed that CTGF could promote the recovery of inured anterior cruciate ligament. It can upregulate the mRNA and expression of TGF-β1, type 1 collagen (COL-I), type 2 collagen (COL-II), Sox9 as well as the tissue inhibitor of metalloproteinase-1 (TIMP-1), and also down-regulate the mRNA and expression of matrix metallopeptidase 13 (MMP-13). These results confirmed the curative effect of CTGF on injured rabbit ACL, and the mechanism is through the regulating these TGF-β1 associated cellular factors.

2. Materials and method

2.1. Rabbits

30 healthy male New Zealand white rabbits at age of 6 months were obtained from Medical Experimental Animal Center of Guangdong Province. Animals were housed in specific pathogen-free (SPF) animal house facility at our hospital, which was controlled under 22–24 °C with 50–60% humidity. The animals had easy access to food and water before being used in experiments. The animal use protocol was reviewed and approved by the Institutional Animal Care and Use Committee of Hospital.

2.2. Anterior cruciate ligament surgery in the rabbit

The rabbits were only provided water but not food 12 h before the surgery. 160,000 units of penicillin were given through buttock injections to prevent bacterial infection before surgery. After achieving general anesthesia with an intravenous injection of 3% pentobarbital sodium solution (30 mg/kg), the rabbits were fixed on the operation table, and the left hind leg of the rabbit was shaved, disinfected, and draped. A longitudinal incision about 3 cm in length was made at the medial border of the patellar tendon to expose the knee joint. The fascia and the muscle were carefully separated, and the wound was washed by saline to avoid getting dry. Half of the anterior cruciate ligament was cut, and the rest half was still connected to tibia and femur.

The 30 New Zealand rabbits were randomly divided into two groups, groups A (control group) and B (treatment group), each with 15 rabbits. Rabbits in A group were given 0.5 ml fibrin gel which was inserted between the bone and ligament near the entrance of femoral tunnel. Rabbits in B group were given 0.5 ml fibrin gel containing15 ng CTGF which was inserted in the same position. Then the wound was sutured in layers and then cleaned by iodine, followed by penicillin power covering and bondage wrapping.

2.3. Postoperative animal care

The rabbits were given penicillin at a dose of 160,000 units per days for 3 continuous days after the surgery, to prevent infection in the operated knee, which was immobilized in extension using an elastic bandage for a period of 5 days post-surgery. All rabbits were allowed to move freely and resumed normal activity 2 days after the surgery. General examination of these animals was performed daily to detect any clinical sign of pain and other complications such as anorexia, abnormal cry, decreased activity, and leg dressing problems. Dermal sutures out was done 7 days after surgery.

2.4. Anatomical observation

2 weeks after operation, the rabbits were sacrificed by air embolism at the edge of the ear vein, and the knee joints of groups A (control group) and B (treatment group) rabbits were obtained to be observed.

2.5. Specimen collection

To determine the blood concentration of basic fibroblast growth factor (bFGF) and TGF-β1, 1 cc ear vein blood was collected from rabbits of each group on the 15th day after model construction. The supernatants in the blood samples were collected after high speed centrifugation, and the levels of bFGF and TGF-β1, respectively, were determined with corresponding ELISA kits (R&D Systems), following the manufacturer's instructions.

2.6. Quantitative real-time PCR

The rabbits in both groups were sacrificed by air embolism on the 15th day after surgery. 6 of 15 rabbits in each group were selected for further analysis. The ligament tissue samples were isolated and frozen in liquid N_2. When needed in experiment, they were taken out from liquid N_2, washed with phosphate buffered saline (PBS), and sliced into small pieces. Total RNA (2 μg) was extracted using ISOGEN (Nippon Gene, Tokyo, Japan) and was subjected to RT-PCR Quantitative real-time PCR was performed with the IQ5System (Bio-Rad). PCR reactions were performed in 25-μl reactions with SYBR® Green Real-time PCR master mix (Toyobo, Japan) and 0.2 μmol/L specific primers. Primer sequences are shown in Table 1. PCR was performed by incubation for 2 min at 95 °C followed by 50 amplification cycles with a 20-s denaturation at 95 °C, 30-s annealing and 30-s extension at 72 °C.

2.7. Western blotting

Rabbit ligament tissue lysates were fractionated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)

Table 1
Primer sequences used for real-time PCR analysis.

Gene	Forward primer Reverse primer
TGF-β1	GTGCGGCAGTGGTTG AGC GGTAGTGAACCCGTTGATGTCC
COL-I	CGACCTGGTGAGAGAGGAGTTG AATCCATCCAGACCATTGTGTCC
COL-II	AACGGTGGCTTCCACTTC GCAGGAAGGTCATCTGGA
TIMP-1	GGCTTCTGGCATCCTGTTGTTG AAGGTGGTCTGGTTGACTTCTGG
MMP-13	AGGAAGACCTCCAGTTTGCAGAG GCTGCATTCTCCTTCAGGATTC