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 beta 1 (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 gene 9 (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 injured 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 CTGF’s healing role in injured tissues and provided new possibilities of treating injured tissues and wound healing by using CTGF.

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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).

CTGF, also known as CCN2 (Jun and Lau, 2011; Hall-Glenn and Lyons, 2011), is a matricellular protein of the CCN family of
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 CTFG was associated with wound healing and virtually all fibrotic pathology (Leask and Abraham, 2006; Brigstock, 2010). Recently, people found that TGF-β1 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 CTFG could promote the recovery of injured 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 metalloproteinase 13 (MMP-13). These results confirmed the curative effect of CTFG 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 buttotck 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 containing 15 ng CTFG 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 check 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 N2. When needed in experiment, they were taken out from liquid N2, 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 IQ™System (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)
and detected by immunoblotting. Briefly, proteins separated with 7% SDS-PAGE were transferred onto PVDF membranes for 45–80 min at 100 V. The membranes were blocked with TBST (TBS/0.5% Tween-20) containing 5% non-fat dry milk for 3 h at room temperature, and were then incubated with 1 polyclonal antibodies (Abcam, UK) reacting on rabbit TGF-b1, COL-I, COL-II, TIMP-1 and MMP-13 at 4 °C overnight, respectively. The membranes were washed four times with TBST, and incubated with 1:3000 diluted HRP-conjugated corresponding secondary antibodies (Boster, China) at 37 °C for 1 h. After being washed four times with TBST, the immunoreactive traces were detected with ECL Kit (Millipore, USA). β-actin was used as a control to determine the sample loading size and also to validate the experiment settings. The intensity of each protein expression on the membranes was scanned by Alphalimage scanner and analyzed by AlphaEase FC image process software (Alpha Innotech).

2.8. Hematoxylin and eosin staining and observation

The frozen tissue is sectioned in cryostat (a sectioning microscope in a freezing chamber) and placed on a microscope slide for staining. The section is fixed immediately using 10% buffered formalin before it begins to decay and is then stained using hematoxylin and eosin stain (HE) which is a routine staining method in clinical (Chan, 2014). After HE staining, the section was carefully observed with an automated digital system, Cytation 5 Cell Imaging Multi-Mode Reader (Biotek, USA).

2.9. SEM analysis

The ligament tissues of rabbits in 2 groups were fixed with 2.5% glutaraldehyde phosphoric acid buffer and dehydrated with a series of ethanol, then vacuum dried. The surface of the specimen was sprayed with gold and observed by scanning electron microscope (Philips XL30E SEM).

2.10. Statistical data analysis

Statistical analysis was performed with the program SPSS statistical software, version 19.0 (IBM Corp., Armonk, NY). Data were expressed as the mean ± standard deviation (SD). Statistical analyses were performed with two-tailed student t-test for two groups comparison. Statistical significance was assumed at p < 0.05.

3. Results

3.1. General observation

The rabbits were observed closely during the whole experiment. Before the surgery, the rabbits in both groups exhibited peaceful and healthy mood, with normal diet and sensitive reaction to changes in the surrounding environment. However, in the first week after the surgery, the rabbits in both groups exhibited serious malaise, and insensitive to environment change, with reduced diet. The original white hair started to become yellow, and even partial alopecia. The rabbits moved much less with reduced speed. Later in the following week, the rabbits in A group remained as before, but the rabbits in B group gradually became better and better, exhibiting a close to normal health condition. Before the surgery, the rabbits in both groups exhibited peaceful and healthy mood, with normal diet and sensitive reaction to changes in the surrounding environment. However, in the first week after the surgery, the rabbits in both groups exhibited serious malaise, and insensitive to environment change, with reduced diet. The original white hair started to become yellow, and even partial alopecia. The rabbits moved much less with reduced speed. Later in the following week, the rabbits in A group remained as before, but the rabbits in B group gradually became better and better, exhibiting a close to normal health condition. The observation demonstrated an overall effect of CTGF in injured ACL recovery.

3.2. Anatomical observation

The right knee joints (normal) of A, B group rabbits had no swelling and hyperemia and the fascia is intact. The muscle was soft, ruddy color, no sense of tension. Ligaments were shiny, without adhesions. The joint capsules became non-thickened, pink, no obvious edema and hyperaemia. Joint fluid was clear, less quantity. Articular cartilage wasn’t defective and bright, and also had a smooth surface.

The left knee joints of A group rabbits were markedly swollen with partial hyperemia. Fascia appeared defects, incomplete. Muscles became tense with mild atrophy and their color became dark. Ligaments were lackluster, thinner than those of right knee joints. The surrounding tissue appeared a certain degree of adhesion. The joint capsules became thickened and the synovial membranes were obviously proliferated with the visible hyperemia. Joint fluid was turbid and its amount increased. Articular cartilage was defective, lackluster. Some of the joints were observed in osteophyte formation.

Compared with A group rabbits, the left knee joints of B group rabbits were mild swollen and the degree of fascia defect is lighter. The state of the muscles and ligaments were similar with those in right knee. Joint capsule showed mild thickening and synovial hyperplasia reduced. The amount of Joint fluid was a little more than that in right knee. Articular cartilage was mildly defective but no joint osteophyte formed. These results showed that connective tissue growth factor can promote the healing of ACL in this experiment.

3.3. Concentration of bFGF and TGF-β1 in the blood

The amount of bFGF and TGF-β1 in the blood of rabbits of both group A and group B was detected by ELISA, and the results were summarized in Table 2. Both the bFGF and TGF-β1 in the group B rabbits blood exhibited a much higher concentration than that in the group A rabbits blood (p < 0.05), indicating that CTGF treatment increased the release of both bFGF and TGF-β1 in the blood.

| Group | bFGF (ng/ml, X ± SD) | TGF-β1 (ng/ml, X ± SD) |
|-------|----------------------|------------------------|
| A     | 48.42 ± 3.75         | 20.64 ± 2.79           |
| B     | 69.49 ± 3.85*        | 46.74 ± 5.95*          |

*: Comparing to the value in group A, the difference is significant (p < 0.05).
3.4. mRNA level of TGF-β1, COL-I, COL-II, Sox9, TIMP-1 and MMP-13

Using RT PCR, we measured the mRNA level of the important cellular factors including TGF-β1, COL-I, COL-II, Sox9, TIMP-1 and MMP-13. As shown in Fig. 1, compared to the mRNA level of rabbit in group A, the mRNA level of TGF-β1, COL-I, COL-II, Sox9 and TIMP-1 in group B were all significantly higher (p < 0.05) and were respectively 1.32 ± 0.64, 0.57 ± 0.14, 0.94 ± 0.12, 0.87 ± 0.07, 0.98 ± 0.16, which suggests CTGF can upregulate these genes’ mRNA level. On the contrary, the mRNA levels of MMP-13 in the ligament tissue of group B rabbits was lower than that in the group A rabbits (p < 0.05), which indicates that CTGF can lower the protein expression of MMP-13.

3.5. Protein levels of TGF-β1, COL-I, COL-II, TIMP-1 and MMP-13

Western blotting was used to measure the protein levels of the important cellular factors including TGF-β1, COL-I, COL-II, TIMP-1 and MMP-13. As shown in Table 3 and Fig. 2, the protein expression level of TGF-β1, COL-I, COL-II and TIMP-1 in the ligament tissue of group B rabbits were all higher than that in group A rabbits (p < 0.05), demonstrating that CTGF increased the protein expression of these four factors. On the other hand, however, the protein level of MMP-13 in the ligament tissue of group B rabbits was lower than that in the group A rabbits (p < 0.05), which indicates that CTGF can lower the protein expression of MMP-13.

3.6. Pathological change observed by HE staining

Pathological changes in the tissues in rabbits of each group were compared after HE staining, seen in Fig. 3. The collagen fibers in the ligament tissue of the rabbits in A group exhibited disordered arrangement, with a loosen microstructure and many broken collagen fibers. Fibroblasts were distributed unevenly, with significantly increased number of nuclei, which underwent some shrinkage changes. However, the collagen fiber in the ligament tissue of the rabbits in group B formed a wave-like structure with no broken collagen fibers. Fibroblasts increased in numbers, but there was no visible changes in the nucleus. Fibroblasts exhibited regular oval shapes and evenly distributed, which represents a recovered ligament tissue.

3.7. SEM images

Further pathological changes in the tissues in rabbits of each group were compared by scanning electron microscope, seen in Fig. 4. We observed that there was less fibroblasts and only a small amount of collagen fibrils in the ligament tissue of the A group rabbits, which suggest that the ability of cell proliferation was weak. However, for the B group rabbits, fibroblasts and collagen fibrils were significantly increased because of strong proliferative ability, which represents a recovered ligament tissue.

4. Discussion

Basic fibroblast growth factor (bFGF), also referred to as FGF-2, is a representative growth factor which has shown the potential effects on the repair and regeneration of tissues (Delgado-Rivera et al., 2009; Hankemeier et al., 2005; Gohar et al., 2017). It has been
reported that TGF-β1 could induce proliferation in human renal fibroblasts by mean of induction of bFGF (Khalil et al., 2016; Strutz et al., 2001). In our study, we observed that CTGF treatment in the injured ACL increased the blood concentration of both TGF-β1 and bFGF. Probably CTGF first stimulated the secretion of TGF-β1, which then further induced the production of bFGF.

The repair and remodeling of connective tissue involves not only chondrocyte reproduction and activity but also the formation of collagen fibers and ground substance. Both COL-I and COL-II are important nature resources for tissue regeneration and wound healing (Huang et al., 2015; Oliveira et al., 2009). In our study we found that CTGF has stimulated the production of COL-I and COL-II in the injured ACL, which is needed for the ACL reconstruction and the ligament recovery.

Matrix metallopeptidase 13, also known as Collagenase 3, is a member of the matrix metalloproteinase (MMP) family involved in the breakdown of extracellular matrix (Vine et al., 2015; Hijoja, 2005). The preferred substrate for MMP-13 is collagen II, which is cleaved five times faster than collagen I and six times faster than collagen III (Mitchell et al., 1996), and more readily by MMP-13 than by other collagenases. In our study, we found that COL-I and COL-II were both increased in B group rabbits, which is in accord with the fact that the MMP-13 level were greatly reduced in group B rabbits. The tissue inhibitor of metalloproteinase-1 (TIMP-1), a 28.5 kDa glycoprotein that belongs to the TIMPs family, is one of the endogenous inhibitors of MMPs (Wu et al., 2008). We found that both the mRNA and protein levels of TIMP-1 were increased in group B rabbits, and also decreased level of MMP-13 mRNA and protein levels. This confirms the inhibition role of TIMP-1 on MMP-13, which is helpful in maintaining sufficient collagens for ACL reconstruction and ligament recovery (Zhao and Ashraf, 2016; Zaidi et al., 2017).

5. Conclusions

Our current study showed that Connective Tissue Growth Factor can promote the recovery of injured anterior cruciate ligament. It can upregulate the mRNA and expression of TGF-β1, COL-I, COL-II as well as TIMP-1, and down-regulate the mRNA and expression of MMP-13. Our findings demonstrated that the curative effect of CTGF on injured rabbit ligament is through regulating these cellular factors.

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