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Mechano growth factor-C24E, a potential promoting biochemical factor for ligament tissue engineering

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ABSTRACT

Ligaments play an important role in stabilizing and balancing knee joint movement. Anterior cruciate ligament (ACL) injuries remain a challenge in clinical and experimental settings due to the poor healing potential of ACL injuries. This study investigates whether the short peptide Mechano growth factor-C24E (MGF-C24E) is an efficient biochemical factor to promote repair of ACL injuries. We found that MGF-C24E-treated (5–30 ng/ml) synovial fibroblasts (SFs) subjected to the static stretching (12% strain) were experienced significant lower oxidative stress, endoplasmic reticulum stress, and the activity of matrix metalloproteinases (MMPs) by SFs was also decreased. In addition, when ACL fibroblasts (ACLFs) were injured and then cultured in SFs culture medium after the SFs had been treated with MGF-C24E, the ACLFs exhibited increased proliferation, migration, collagen synthesis and enhanced mechanical recovery. These results indicate that MGF-C24E can interact with injured SFs and affect the injury microenvironment of the knee to promote ACLFs mechanical injury repair.

1. Introduction

The anterior cruciate ligament (ACL) is a ligament that attaches to the front of the intercondyloid eminence of the tibia, and is blended with the anterior horn of the medial meniscus. This structure allows the ACL to resist anterior translation and to ensure medial rotation of the tibia, thus stabilizing the knee joint. Injured ACL can cause knee instability, pain, and progressive degeneration of other knee joint structures, such as osteoarthritis [1]. The ACL is covered by a thin layer of tissue called the synovium that is involved in secretion and resorption of synovial fluids. These fluids bring the joint nutrients and remove waste, directly affecting the knee cavity environment. In addition, synovial fluids seep into microcavities and form a layer on the cartilage surface to reduce friction and absorb shocks to the joints [2].

Over the last decade increased participation in sports has correlated with increased number of joint injuries, especially to the ACL. Certain sports are more likely to lead to long-term joint injuries and ACL injury may leave a person more susceptible to other injuries [3]. When the ACL is mechanically injured, the synovial membrane can also be injured, due to the particular location of the ACL in the joint and low blood flow to the area. Synovial fluid can produce various proteolytic enzymes that influence the joint cavity microenvironment. Scar tissue from an injured ACL may be difficult to bend and may lack the capacity to produce an inflammatory response. Therefore, the ACL has long been thought to have poor healing capacity, with a substantially high rate of failure (40–100%), even after surgical repair using sutures [4,5].

Researchers in ligament tissue engineering are especially interested in testing whether the articular cavity microenvironment can be adjusted to promote the repair of a damaged ACL. Since the ACL is covered by synovium, it is difficult to access injured ACL during surgery. But the synovium plays an important role in response to trauma, as well as in inflammatory and degenerative disorders of the joints. The synovium can also regulate blood flow to release substances such as catecholamines and serotonin to control metabolism in knee joints during healing [6]. Our previous study has demonstrated that, when ACL was injured, synovial fibroblasts were involved in regulating the microenvironment of joint

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cavity, which affects the activity of MMPs and production of lysyl oxidases (LOXs) in ACL. Early interventions to inhibit the activity of MMPs or promote the production of LOXs in the synovial fibroblasts should be performed to facilitate the healing of ACL [7,8]. In sum, the synovium has an irreplaceable function in ACL injury repair and mimicking the synovium-ACL microenvironment may promote ACL repair. However, effectively reproducing the positive microenvironment of the synovium remains a challenge [9].

Recent studies have reported that growth factors can improve patient outcomes after the surgical repair of ACL injuries. Growth factors have been tested for orthopedic treatments such as fracture management, spinal fusion and tendon and ligament healing. Growth factors like transforming growth factor-beta (TGF-β) [10], platelet derived growth factor (PDGF) [11] and vascular endothelial growth factor (VEGF) [12] show the most promising results for healing ACL injuries [13]. Biochemical factors that can be added to tissue-secreted proteins may offer opportunities to improve ACL injury repair and to promote regeneration in clinical settings.

MGF-C24E is one of the alternative splicing products of insulin-like growth factor 1 (IGF-1) and plays a pivotal role in musculoskeletal injury repair [14]. MGF-C24E is formed when total IGF-1 mRNA contains 6 exons and can be translated into a precursor that cleaves to form the N-terminal peptide named mature IGF-1, and a C-terminal fragment named E-peptide. IGF-1 splice variants of exon 4 spliced to exon 5 or exon 6 are named MGF or IGF-IEc in humans and IGF-IEb in rodents [15–17].

As a growth factor that is sensitive to mechanical stress, MGF-C24E has been shown to have a surprising effect on tissue repair by promoting cell migration, proliferation and buffering the cells against stress [14]. It has been reported that MGF-C24E can increase the pool of satellite cells and muscle progenitor cells for muscle injure repair. What's more, MGF-C24E can promote tenocyte migration to assist tendon repair and can be used as a neuroprotective by adjusting oxidative stress as well [18–20]. The effects of MGF-C24E on ACL repair have never been tested.

When mechanical injury occurs, both ACL and synovium will undertake mechanical stretch and injury since ACL is covered by synovium. During the injuring process, ACL is mechanically injured, the synovial membrane also undertake similar strain, disorders of cell stress and proteolytic enzymes activity in synovium tip the balance of knee cavity microenvironment, which eventually leads to ACL fail to heal. Thus, we hypothesize that, when synovium and ACL undergone mechanical injury, MGF-C24E can relieve cell stress and regulate proteolytic enzymes activity of synovial fibroblasts, which provides a positive microenvironment for ACL to heal. In order to mimic the process of ACL injure, both SFs and ACLFs were statically stretched by FX-4000 Flexercell Tension Plus System. On one hand, cell stresses, deformation of cell and MMP activity were detected during synovial fibroblasts undertake static stretching and MGF-C24E treatment. On the other hand, when ACL undertake static stretching, and co-cultured with synovial fibroblasts supernatant, the ACL repair capacity was investigated through MMP expression analyses, collagen synthesis, cell migration, proliferation and viscoelasticity. The present study aims to pave new avenue in repairing ACL injury by short peptide.

2. Materials and methods

2.1. Cell culture

Human synovial fibroblasts (SFs) and ACL fibroblasts (ACLFs) were donated by K.L. Paul Sung (University of California, San Diego). Cells were harvested from four donor tissues (between the ages of 30–60, two male and two female subjects) undergoing limb amputation at First Affiliated Hospital of Chongqing Medical University, Chongqing, China. The standard operating procedure of synovium fibroblasts and ACL fibroblasts culturing was described in an earlier study [5]. Cells harvested from passage 3 to passage 5 were used for experiments.

2.2. Cell co-culture

For the cell co-culture study, ACLFs were cultured with co-culture medium, consisting of 2% FBS DMEM culture media and SFs supernate (1:1). These co-culture medium including SFs supernate, SFs without any treated (SFs supernate); SFs supernate, after SFs undergoing static stretch, but without MGF-C24E treatment (SFs-stretch supernate); or SFs supernate, SFs with MGF-C24E added during mechanical stretching (SFs-stretch-MGF supernate). ACLFs were incubated with one of the co-culture mediums and subjected to a 12% static stretching for 12 h. Monolayer cultured ACLFs without any treatment act as the Sham.

2.3. MGF-C24E treatment and static stretch

For each experiment, cells were trypsinized and resuspended in DMEM culture medium (Gibco, 11965-084, USA) with 10% fetal bovine serum (FBS; Gibco, 16000-044, USA). The cells were then reseeded uniformly into flexible-bottom 6-well culture plates coated with Collagen Type I (BioFlex® Culture Plates BF-3001C, Flexcell Inc., USA) at a density of 5×10^4 cells per well, and were allowed to adhere and equilibrate over 48 h. When cells attained 90–95% confluence, the culture media was removed and replaced by 2% FBS media for 16 h of starvation. The media was removed and replaced with fresh 1% FBS media containing MGF-C24E (0, 5, 10, 20 and 30 ng/ml, Phoenix Pharmaceuticals Inc., USA) and then diluted with DMEM culture medium. Both SFs and ACLFs during injury conditions in vitro were mimicked by using the FX-4000 Flexercell Tension Plus System (Flexcell Inc. Hillsborough, NC, USA). SFs or ACLFs cultured in the flexible bottom of 6-well culture plates were exposed to static stretching with 12% strain and frequency of 1.0 Hz for 12 h [21]. SFs and ACLFs were monolayer cultured but without static stretching and MGF-C24E or co-culture treatment act as Control and Sham respectively. This experiment was repeated at least for 3 times.

2.4. Quantitative real-time polymerase chain reaction (qRT-PCR)

Quantitative real-time PCR was performed with the SsoAdvanced SYBR Green PCR supermix (1725264, Bio-Rad) using iCycler (Bio-Rad) according to described techniques [22]. The cDNA were collected from different SFs experimental groups including the Control, SFs undertake static stretching group (SFs + stretch) and MGF-C24E-treated SFs subjected to the static stretching group (SFs + stretch + MGF). PCR reactions were performed in a 25 μl volume with 0.5 μl of each primer and 1 μl cDNA sample. The reaction was initiated by activating the polymerase with a 5 min pre-incubation at 95 °C. Amplification was achieved with 40 cycles of 15 s denaturation at 95 °C, 1 min annealing at 60 °C and 10 s extension at 72 °C. BLAST was used to search for all of the primer sequences to ensure gene specificity. Selected sets of primers are shown in Table 1 and were previously reported [23,24]. This experiment was repeated for 3 times.

2.5. Measurement of intracellular ROS production

Intracellular ROS levels were measured by assessing the oxidative conversion of cells permeable to DCFH-DA (Beyotime Institute of Biotechnology, China) and fluorescent dichlorofluorescein (DCF) by fluorescence microscope. Approximately 1×10^5 cells were seeded onto flexible-bottomed 6-well culture plates for 48 h