

ORIGINAL PAPERS

Impact of chronicity of injury on the proportion of mesenchymal stromal cells derived from anterior cruciate ligaments

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Abstract

Background aims. The graft-healing potential of mesenchymal stromal cells (MSCs) derived from the remnants of ruptured anterior cruciate ligaments (ACLs) after ACL reconstruction may depend on the chronicity of the injury. The aim of this study was to assess the quantitative and phenotypic differences between MSCs isolated from ACL remnants in patients with (sub) acute and chronic tearing. **Methods.** Torn ACL remnants were harvested during ACL reconstruction from 41 patients, 24 with (sub)acute ACL (<6 months from injury to surgery) and 17 with chronic ACL (time interval >6 months) tears. MSCs isolated from these samples were assessed for quantitative and phenotypic differences, and the correlation between the proportion of MSCs and the chronicity of ACL tear was evaluated. **Results.** At passage 0, the mean proportion of MSCs (CD34⁺, CD44⁺, CD90⁺ and CD105⁺) was higher in (sub)acute than in chronic ACL tear samples (20.69% ± 7.82% versus 9.85% ± 8.01%, $P < 0.001$). At passages 1 and 2, however, MSC proportions did not differ significantly in the two groups. Time interval showed a negative correlation with MSC proportion only at passage 0 ($r = -0.505$, $P < 0.001$). The optimal cutoff value for time from injury to surgery yielding <10% freshly isolated ACL-MSCs, a percentage expected to have low tissue healing potential, was 23.5 months. **Conclusions.** The proportion of freshly isolated MSCs was higher in samples from patients with (sub)acute tearing than in chronic ACL tearing and negatively correlated with the time interval between trauma and surgery.

Key Words: acute ACL, anterior cruciate ligament, chronic ACL, mesenchymal stromal cells

Introduction

Anterior cruciate ligament (ACL) reconstruction is one of the most common orthopedic surgeries performed around the world. Proper reconstruction of a ruptured ACL, however, does not guarantee good results, with success rates ranging from 80–90% (1,2). In addition, persistent knee pain and instability have been reported in 10–30% of patients with poor long-term outcomes (3). New surgical techniques, such as double-bundle reconstruction and anatomical reconstruction with the use of the anteromedial portal, have been developed as alternatives to single-bundle ACL reconstruction with the transtibial technique, especially to improve rotational instability. However, modifications of these surgical methods have raised

concerns about potentially higher complication rates. Neither the double-bundle nor the anatomical single-bundle reconstruction method has produced better long-term clinical outcomes than the single-bundle transtibial technique. In addition, the success of ACL reconstruction may depend not only on mechanical stability but on biological graft healing (4).

Considerable attention has been focused on the role of remnants of a ruptured ACL in reconstruction. These remnants may enhance revascularization and cell proliferation as well as promoting the recovery of proprioception (5). Therefore, preservation of these remnants may improve the results of the standard ACL reconstruction technique (the single

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bundle with transtibial technique) by enhancing biological graft healing.

The inherent characteristics of mesenchymal stromal cells (MSCs), which include self-renewal and multilineage differentiation potential, make these cells promising agents for use in tissue repair and the regeneration of damaged tissue. Increasing evidence suggests that MSCs are not exclusive to the bone marrow but are also present in various tissues. MSCs have been isolated from intra-articular tissues, including the synovial membrane, synovial fluid, articular cartilage, and ACL (6–8). The promising healing-promoting properties of MSCs and their presence in the ACL (8,9) suggest that MSCs in the remnants of a ruptured ACL may promote graft healing after ACL reconstruction. The proportion of *ex vivo* MSCs cultured from ACL remnants is important for effective tissue preparation, and this proportion may depend on donor-related factors, in particular, the time interval between ACL rupture and surgery (chronicity). An over-long delay may result in wear and thinning of remnant tissue, leading to less favorable results. To date, no studies have directly compared MSC proportions derived from ACL remnants (ACL-MSCs) in patients with (sub)acute and chronic ACL tears, either immediately after isolation or during culture. Moreover, it is unclear whether the proportion of MSCs in ACL remnants is correlated with the chronicity of ACL tearing. We hypothesized that the proportion of MSCs in ACL remnants may differ in patients with (sub)acute and chronic ACL tears and that this proportion may be correlated with chronicity. We therefore assessed the quantitative and phenotypic differences between MSCs isolated from ACL remnants in patients with acute tearing and chronic tearing. We also evaluated the correlation between MSC proportion and the chronicity of ACL tearing.

Methods

Study design, preoperative evaluation and collection of ACL remnants

This prospective, longitudinal trial enrolled all candidates for ACL reconstruction with isolated ACL ruptures confirmed by magnetic resonance imaging (MRI) and physical examinations, such as positive anterior draw and Lachmann and/or pivot shift tests (greater than grade II). We excluded patients with other concomitant intra-articular (ie, meniscus or ligament injuries) or associated extra-articular lesions and those who were undergoing revisional ACL reconstruction to exclude any biases arising from other potential sources of MSCs on the proportion of MSCs in ACL remnants. Torn ACL remnants were

harvested with pituitary forceps during ACL reconstruction. Patients who underwent surgery <6 months after injury were categorized as having (sub)acute ACL tears, whereas those who underwent surgery after 6 months were categorized as having chronic ACL tears. Furthermore, the classification of each patient as having a (sub)acute or chronic ACL tear was predicted by preoperative MRI and confirmed arthroscopically. The ethical approval of this study protocol was granted by institutional review board of our institution. Written informed consent was obtained from all subjects before participation in this study.

Cell isolation and culture

Each ACL sample was weighed, washed twice with $\times 1$ phosphate-buffered saline (PBS), minced into small pieces about 1 mm in size and incubated with 3% collagenase type I (Worthington Biochemical Corp, Lakewood, NJ, USA) in $\times 1$ PBS for 2 hours at 37°C. The suspension was washed with $\times 1$ PBS and centrifuged at 1500 rpm for 10 min and the supernatant was discarded. Each pellet was resuspended in 50 mL $\times 1$ PBS and passed through a 70- μ m nylon cell strainer (BD Falcon 352350; BD Biosciences, Bedford, MA, USA) with the use of a 15-mL syringe plunger to remove clumps. The resulting single-cell suspensions were centrifuged at 1500 rpm for 10 min and the supernatants were discarded. Red blood cells in each sample were lysed by incubation with 1 mL ammonium-chloride-potassium lysing buffer (150 mmol/L NH_4Cl , 10 mmol/L KHCO_3 , 0.1 mmol/L Na_2 ethylenediamine tetra-acetic acid [EDTA], pH 7.2–7.4) for 5 min at room temperature (RT). The tube was topped with $\times 1$ PBS, and the cells were filtered through nylon mesh, centrifuged at 1500 rpm for 5 min and resuspended in Poietics Mesenchymal Stem Cell Growth Medium (MSCGM Bulletkit PT-3001; Lonza Walkersville, Inc, Walkersville, MD, USA). Cells were counted by Trypan blue exclusion, and 1×10^5 cells in 10 mL of MSCGM were seeded in a 100-mm-diameter culture dish and incubated for 14 days in a humidified 5% CO_2 incubator at 37°C. The attached cells were harvested with 1 mmol/L EDTA in PBS, transferred to a 15-mL conical tube (SPL Lifesciences, Pocheon, Gyeonggi-do, South Korea) and centrifuged at 1500 rpm for 5 min for further analyses.

Flow cytometry

Flow cytometry assays were performed to characterize the ACL-derived MSCs. Cultured cells were detached from the culture dishes with 1 mmol/L EDTA in PBS, centrifuged at 1500 rpm for 5 min and resuspended in 1 mL of MSCGM. After counting, 1×10^5 cells were transferred to a polystyrene

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