Age-related Differences in Anterior Cruciate Ligament-derived Cells

Atsu Uefuji, Tomoyuki Matsumoto, M.D., Ph.D., Takehiko Matsushita, Takeshi Ueha, MS, MOT, Zhang Shurong, Masahiro Kurosaka, Ryosuke Kuroda.

1Kobe University Graduate School of Medicine, Kobe, Japan, 2Neochemir Inc., Kobe, Japan.

Disclosures:

Introduction: The anterior cruciate ligament (ACL) is the primary stabilizer of anteroposterior knee translation. It is susceptible to injury and does not heal spontaneously after injury owing to poor vascularization of the ligament [1,2,3]. Over the last decade, further knowledge about intrinsic healing potential of the ACL has been found. The stem cell is one of the intriguing new approaches to ACL healing because of its potential for high expansion, self-renewal, and multipotent differentiation capacity. In prior studies in our laboratory, CD34+ cells were found to be developmentally and anatomically related to blood vessel walls in human tissue. And these CD34+ cells exhibit multilineage potential, both in culture and in vivo, into skeletal myofibers, bone, cartilage, adipocytes, and endothelial cells [4,5]. ACL-derived CD34+ cells contribute to tendon-bone healing, but the relationship between age and ACL healing potential has not been clarified. In human bone marrow, proliferation and osteogenic differentiation potential was shown to decline by aging [6]. Therefore, the present study was designed to test the hypothesis that there are age-dependent differences in the number of CD34+ cells, proliferation, and multilineage differentiation potential in ACL remnant tissue.

Methods: Ruptured ACL remnants were harvested from 28 patients (mean age, 24.6 ± 1.6 years) who underwent primary arthroscopic ACL reconstruction. The three patients groups divided by age were: 10s group (n = 10), 20s group (n = 10), and 30s group (n = 8). Human ACL tissue was transported in sterile saline solution on ice. A part of ACL remnant was frozen and stored at -80°C. Six-µm sections were prepared for hematoxylin and eosin (H&E) staining for identifying the remnant tissue’s structure. Another part of ACL remnant was digested to cells with 0.4% collagenase type II. The ACL remnant cells were characterized using fluorescence-activated cell sorting (FACS) for CD34, CD146, CD45, CD44, CD29, and Stre-1 expression. Expansion potential was evaluated using population doubling (PD), and multilineage differentiation potential was assessed and compared in osteogenic differentiation, endothelial differentiation, chondrogenic differentiation, and adipogenic differentiation. Osteogenic differentiation was assessed using alkaline phosphatase (ALP) staining, alizarin red staining, ALP activity and gene expression of osteocalcin, ALP, and Runt-related transcription factor 2 (Runx2). Endothelial differentiation was assessed using acLDL/EUA staining cell count, tube length and gene expression of VE-cadherin and CD31. Chondrogenic differentiation was assessed using toluidine blue staining and safranin O staining. Adipogenic differentiation was assessed using oil red O staining. The data were analyzed statistically using ANOVA with post-hoc test. P<0.05 was considered to be significant.

Results: H&E staining demonstrated blood vessel-like formation in the ACL remnant tissue. These structures were confirmed as blood vessels by α-SMA staining (green). CD34+ cells (red) were found in abundance around the blood vessels’ regions. FACS analysis showed numerous CD34+ cells in the 10s compared with the 30s group (25.4% ± 7.9% vs. 16.9% ± 3.9%; p = 0.044) [Fig.1]. PD results indicated that the 10s group had a significantly higher expansion potential than the 30s group in passage 3 (10s: 3.3 ± 0.2 vs. 30s: 2.8 ± 0.2; p = 0.039) [Fig.2]. In osteogenic differentiation, the staining by alizarin red and ALP was better in the 10s group, and an age-related decline was observed in the groups [Fig.3A]. Adolescent ACL remnant cells had a higher potential according to ALP activity (10s: 169.5 ± 37.9 × 10 ng/mL vs 30s: 64.9 ± 14.6 × 10 ng/mL; p = 0.029) and osteocalcin gene expression (10s: 1.0 ± 0.25 vs 30s: 0.39 ± 0.01; p = 0.01) [Fig.3B]. In endothelial differentiation, the 10s group displayed higher differentiation potential than other groups in acLDL/EUA-stained cell counts (10s: 15.9 ± 1.9 vs 20s: 8.9 ± 1.3; p = 0.04; 10s group, 15.9 ± 1.9 vs 30s group, 7.2 ± 1.5; p = 0.008) [Fig.4A], and tube length (10s: 6,939 ± 470 µm vs 30s: 4,119 ± 507 µm; p = 0.009) [Fig.4B]. There was no significant difference in gene expression of VE-cadherin and CD31 [Fig.4C]. In chondrogenic differentiation, 10s group showed better staining by toluidine blue and safranin O in compared with the other groups. In adipogenic differentiation, 10s group showed better staining by oil red O in compared with the other groups.

Discussion: We found that CD34+ cells were more prevalent in adolescents’ ACL remnants and decreased with age. Adolescent patients also exhibited high proliferation and multilineage differentiation potential, especially in osteogenic and endothelial differentiation. The importance of ligament and tendon-bone healing is the ability to differentiate into osteogenic and endothelial lineages. As proof of principle, stem cell-like cells in ruptured ACL tissue differentiate into osteoblasts and endothelial cells. In the present study, as additional evidence, we clarified that the healing potential of ACL remnant tissue is reduced with age. Therefore, surgeons should consider the patient’s age while performing remnant-preserving ACL.
reconstruction for predicting healing potential.

**Significance:** During remnant-preserving ACL reconstruction, surgeons should consider the patient’s age when predicting healing potential.

**Acknowledgments:**

**References:**
Fig 1: ACL-derived cells were characterized by FACS for CD34, CD146, CD45, CD44, CD29, and Stro-1 expression rate. The expression rate of CD34+ cells decreased with increasing age, there was a significant difference between the 10s and 30s groups.
Fig. 2: The number of population doublings (PDs) for each passage. The 10s group had significantly higher expansion potential than the 30s group in passage 3.
Fig 3: In osteogenic differentiation, Alizarin red and ALP staining showed better in the 10s group, and an age-related decline was observed in the groups (Fig 3A). In ALP activity and the gene expression of osteocalcin, the 10s group showed significantly higher than the 30s group (Fig 3B).
Fig. 4: The 10s group had significantly higher number of double-positive stained than the 20s and 30s groups (Fig. 4A). The tube length, the 10s group displayed significantly higher potential for tube formation than the 30s group (Fig. 4B). In the gene expression of VE-cad and CD31, there were no significant differences among the 3 groups (Fig. 4C).

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