Enhanced differentiation of mesenchymal stem cells co-cultured with ligament fibroblasts on gelatin/silk fibroin hybrid scaffold

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Introduction: Anterior cruciate ligament (ACL) injury is one of the most common injuries of knee joint. Due to poor vasculization ACL has limited healing capacity. Recently ligament tissue engineering has emerged as a promising remedy. For the easy isolation and pluripotentials mesenchymal stem cell (MSC) is considered as an attractive candidate for tissue engineering [1]. Therefore, a crucial issue is how to induce the differentiation of MSCs towards ligament fibroblasts. Recently co-culture has increasingly become an effective strategy to induce the differentiation of MSCs for its ability to promote cells communications [2]. We hypothesize that the released cytokines from ligament fibroblasts can enhance the proliferation and differentiation of MSCs on scaffold in co-culture system.

Materials and Methods: The MSCs were seeded on silk cable-reinforced gelatin/silk fibroin (G/S) hybrid scaffold at a cell density of 0.5×10⁶ cells/scaffold and transferred to co-culture system. Each MSCs/scaffold was cultured on the transwell insert membrane in 6-well plate and 0.5×10⁶ ACL fibroblasts were cultured in each lower chamber of the well. The MSCs/scaffold cultured without co-culture was used as control. There were two groups in this study, which were co-culture group (Co) and non-co-culture group (N-Co). The DNA amount and the metabolism of MSCs were quantified. Cell viability was observed with confocal laser microscopy and the morphology on scaffold was further examined by scanning electron microscopy. The amount of deposited collagen was also quantified. MSCs/scaffolds from groups were examined with H.E. staining and immunohistochemistry staining for collagen I, collagen III, and tenascin-C. Quantitative real-time RT-PCR and western blot were carried out to quantify the transcription level and protein expression of ligament specific genes including collagen I, collagen III, and tenascin-C.

Results: The DNA content of Co group were 21.8±1.1μg at 1 week and 30.4±3.7μg at 2 weeks, which were significantly higher than that of N-Co group (p<0.05). Although there was no significant difference in metabolism of MSCs on day 1, day 3, and day 5, the value of Co group was significantly higher than that of N-Co group after 7 days. The MSCs were distributed uniformly throughout the scaffold. No significant cell death was found inside scaffold. Furthermore, the MSCs in Co group showed fibroblast-like morphology and abundant ECM. Co-culture dramatically increased collagen production with 99.4±10.3μg at 1 week and 902.2±8.3μg at 2 weeks, which were significantly higher than that of N-Co group (p<0.05). After normalized against DNA content, the value of Co group was still significantly higher than that of N-Co group (p<0.05). Immunohistochemistry staining for collagen I was highly positive in Co group. In comparison with collagen I, collagen III expressed in relatively lower amount and tenascin-C barely expressed in regenerated tissue. The MSCs in Co group were proved to differentiate towards ligament fibroblasts by expressing ligament extra cellular matrix specific genes including collagen I, collagen III, and tenascin-C in mRNA and protein level.

Discussion: Ligament fibroblasts can produce IGF-I, TGF-β, and PDGF. The PDGF can significantly increase cell proliferation. TGF-β can intensively enhance the differentiation and IGF can promote cell survival by up-regulation of IGF signaling pathway. Due to these effects the regulatory signals released from ligament fibroblasts can support the proliferation and differentiation of MSCs. In present study silk cable-reinforced G/SF hybrid scaffold was used to provide 3-D culture environments for MSCs in co-culture system. The maximum load of scaffold was 382.1±8.3 N, which met the ACL force required in normal walking [3]. In co-culture system most growth factors released from ACL fibroblasts are generally basic proteins and they can ionically interact with the negatively charged gelatin of scaffold. This can increase the half-life of growth factors in co-culture system, which is beneficial for the proliferation and differentiation of MSCs on scaffold. In comparison with other methods, such as sequential addition of growth factors and mechanical stimulation, co-culture is proved to be a cost-effective measure to induce the differentiation of MSCs for ligament tissue engineering.


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![Fig.1 Scaffold and schematic of 3-D co-culture system (A) gross observation; (B) scaffold observed by SEM (×150); (C) MSCs/scaffold without co-culture; (D) MSCs/scaffold co-cultured with ligament fibroblasts.](image1)

![Fig.2.MSCs on scaffold in N-Co group and Co group by HE staining and immunohistochemistry staining specific for collagen I, collagen III, and tenascin-C at 2 weeks. (×100)](image2)