Decellularized Tendon-Derived Stem Cell Sheet Exhibited Direct Osteogenic and Immunomodulatory Effects for the Promotion of Graft Healing after Anterior Cruciate Ligament Reconstruction

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Disclosures: Pauline Po Yee Lui holds two patents (U.S. Patent No. 8,945,356, C. N. Patent No. CN 102641546 B) and one provisional patient (U.S. Patent No. 63/268,344) related to the fabrication of the cell sheets in this study.

INTRODUCTION: The outcome of anterior cruciate ligament reconstruction (ACLR) is not satisfactory with graft failure and graft laxity, requiring a second revision surgery. We showed that decellularized tendon-derived stem cell (dTDSC) sheets promoted graft healing in a rat ACLR model. However, the underlying mechanism remains unclear. We have shown that the transplantation of dTDSC sheet promoted tunnel bone formation, angiogenesis, increased M2 macrophages but decreased M1 macrophages, enhanced TIMP1 expression but reduced MMP1 and MMP13 expression. We therefore hypothesized that dTDSC sheet might promote graft healing by enhancing bone formation and modulating the inflammatory environment. This study aimed to examine the osteogenic and immunomodulatory effects of dTDSC sheets in vitro.

METHODS: Animal research ethics committee approved the study. Rat TDSCs were isolated from the Achilles tendons from male Sprague-Dawley rats (6-week-old; weight, 200-220 g) and treated with our proprietary combination of growth factor and biologicals for 2 weeks to induce cell sheet formation. The TDSC was then decellularized according to our published protocol. The content of bioactive factors including SDF-1 and POSTN in the dTDSC sheet and TDSC sheet were compared by ELIZA. MC3T3 pre-osteoblasts were seeded on dTDSC sheet or plastic surface. The viability and proliferation of MC3T3 pre-osteoblasts were examined by AlamarBlue reduction assay and Ki67 mRNA expression while the migration of MC3T3 cells was studied by the transwell assay. The osteogenic effects of dTDSC sheet were examined by calcium nodule formation using Alizarin red S assay and dye quantification at day 28 after induction while the mRNA expression of Bsp, Bglap and Ocx was assessed by qRT-PCR at day 7 after induction. The effects of dTDSC sheet on the polarization and expression of inflammatory cytokines in macrophages were assessed by seeding LPS-treated RAW264.7 cells on the dTDSC sheet or plastic surface for 6 hours. The mRNA expression of C86, CD206, pro-inflammatory (Il1b, Il6, Cxcl10) and anti-inflammatory cytokines (Il10) in macrophages was evaluated by qRT-PCR. The effect of dTDSC sheet in modulating the transcriptomes and hence functions of the treated macrophages was examined by RNA sequencing. The top differential biological processes and signalling pathways were further explored.

RESULTS: dTDSC sheet expressed similar levels of SDF-1 and POSTN compared to the TDSC sheet (p>0.05) (both n=4-6/group), dTDSC sheet increased the viability and proliferation (p=0.050) (n=3/group), expression of Ki67 (p<0.05) (n=6/group), and migration (n=4/group) of MC3T3 cells. More Alizarin red S-stainable calcium nodules (p=0.050) (n=3/group) and higher osteogenic marker expression (all p<0.05) (n=6/group) were observed after seeding MC3T3 cells on the dTDSC sheet compared to seeding on the plastic surface (Figure 1). There was a significant lower mRNA expression of CD86 but higher expression of CD206 after culturing LPS-treated RAW264.7 cells on dTDSC sheet compared to cells seeded on plastics (both p<0.01) (n=6/group) (Figure 2). Seeding of RAW264.7 on dTDSC sheet also suppressed LPS-induced increase of Il1b, Il6 and Cxcl10 as well as increased the expression of Il10 (all p<0.01) (n=6/group). RNA sequencing analysis showed genes that were upregulated in LPS-treated macrophages compared to dTDSC sheet, while the migration of MC3T3 cells was observed after seeding dTDSC sheet compared to cells seeded on plastics. GO analysis showed downregulation of negative regulation and peptidase activities in LPS-treated macrophages seeded on dTDSC sheet compared to cells seeded on plastics. Moreover, processes related to cell division were upregulated as shown in the DEG analysis. Reactome analysis also identified MHC class II antigen presentation as one of the key biological processes upregulated in LPS-treated macrophages seeded on dTDSC sheet compared to cells seeded on plastics.

DISCUSSION: The dTDSC sheet expressed osteogenic and chemotactic factors. It enhanced viability, proliferation, migration, and osteogenic differentiation of pre-osteoblasts. The expression of SDF-1 and POSTN in dTDSCs suggested that it could attract stem/progenitor cells to the injury site and promote their osteogenic differentiation for tissue repair. The osteogenic effect of dTDSC sheet supported increased bone formation at the tendon-bone junction (TBJ) in ACLR after dTDSC sheet transplantation as reported in our previous study. The dTDSC sheet inhibited LPS-induced M1 polarization and enhanced its M2 polarization, with concomitant reduced expression of pro-inflammatory cytokines and increased expression of anti-inflammatory cytokine in macrophages. Transcriptome analysis showed that dTDSC sheet enhanced the proliferation, MHC class II presentation and peptidases activities of LPS-treated macrophages. The findings were consistent with the lower M1 macrophages and higher M2 macrophages observed at the TBJ and graft mid-substance reported previously in our ACLR animal model. The suppression of pro-inflammatory cytokines in macrophages might contribute to higher graft integrity in the dTDSC sheet group compared to the untreated group observed in our ACLR model. Further study is required to identify the bioactive factors in dTDSC sheet and the signalling pathway that promoted M2 macrophage polarization. In conclusion, the dTDSC sheet expressed key growth-promoting factors similar to TDSC sheet. It showed direct osteogenic and immunomodulatory effects, which might explain increased bone formation and lower inflammation and hence better healing outcomes after its transplantation in the ACLR animal model. We will confirm these in vitro findings in the ACLR animal model by immunohistochemistry staining in future.

CLINICAL SIGNIFICANCE: A better understanding of the key biological processes and signalling pathways underlying graft healing effects of dTDSC sheet would provide insight for its optimization and the development of new treatment strategies for the promotion of graft healing after ACLR.

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