MACROPHAGES ACCUMULATE IN THE EARLY PHASE OF TENDON-BONE HEALING

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INTRODUCTION: The natural history and biology of ligament reconstruction using a tendon graft is still not completely understood. Previous studies demonstrate that tendon-to-bone healing proceeds by formation of a fibrovascular tissue at the tendon-bone interface. However, the cells that initiate and regulate the process of tendon-to-bone healing and the temporal sequence of this process are unknown. We have previously reported that the cells in the healing tendon-bone interface proliferate rapidly and may originate from bone-marrow. Several cell types may contribute to tendon-bone interface healing, including cells from vasculature, bone, tendon, marrow, and synovium. We hypothesize that macrophage lineage cells play an important role in early tendon-bone healing. In this study we have used a newly-developed rodent model to identify the cellular response in the early phase of tendon-bone healing.

METHODS: The study protocol was approved by the Institutional Animal Care and Use Committee at our institution. Thirty-six Lewis (inbred) rats (18 male and 18 female) underwent anterior cruciate ligament (ACL) reconstruction in the left knee using a flexor digitorum longus tendon graft. The femoral and tibial bone tunnels were 1.2mm diameter. Both ends of the grafted tendon were secured to the surrounding periosteum using 4-0 Ethibond suture (Ethicon, Somerville, NJ). The tendon donor and recipient were opposite sex (i.e., tendon graft from male donor transplanted into female recipient, and vice versa). Six animals were sacrificed at 4, 7, 11, 14, 21, and 28 days after surgery. Tissue sections were prepared for routine histology and immunohistochemical analysis using the following antibodies: anti-PCNA (proliferating cell nuclear antigen, a marker of active proliferating cells); anti-macrophage antibody (ED1, a marker of recruited macrophages); and anti-Factor VIII (a marker of endothelial cells/blood vessels). Sections were counterstained with hematoxylin. Negative controls were processed in an identical manner except for incubation with bovine serum albumin rather than the primary monoclonal antibody. Serial sections were analyzed to quantify the number of cells that stained positive for anti-PCNA / ED1 and the newly formed blood vessels that stained positive for Factor VIII in three different areas: the tendon-bone interface, the outer tendon, and the inner tendon. A total of 15 high power fields (50 × 50 μm) were analyzed to count the total number of positively-staining cells and total nucleated cells per high power field (Fig. 1). We also compared the similarity between the proliferating cells and macrophages by simultaneously using anti-PCNA antibody and ED1 with a double staining technique (Fig. 2). Statistical comparisons were performed with use of two-tailed Student’s t-test.

RESULTS: There was progressive cell ingrowth from the interface towards the inner tendon. In the tendon-bone interface, PCNA+ cells / ED1+ cells accounted for 71% / 59% of all cells present at 4 days after surgery, and gradually decreased to 26% / 33% at 28 days after surgery. There was no significant difference between the count of PCNA+ cells and ED1+ cells after 11 days. Conversely, PCNA+ cells / ED1+ cells in the inner tendon increased from 5% / 3% at 4 days after surgery to 47% / 44% at 21 days after surgery (Fig. 3, 4). By 28 days after surgery the PCNA+ cells had decreased to 19% but ED1+ cells increased to 59% (Fig. 5). The majority of PCNA+ cells were also ED1+ in the inner tendon at 14 and 21 days after surgery, which was also confirmed with the double staining technique. Findings in the outer tendon were intermediate between the interface and the inner tendon: PCNA+ cells / ED1+ cells were found in the outer tendon at later time points than the interface but at earlier time points than the inner tendon. Newly-formed blood vessels were first found in the tendon-bone interface and then later in the tendon, followed by cellular proliferation within the tendon. Many of the cells within the tendon that were negative for PCNA and ED1 were spindle-shaped, fibroblast-appearing cells.

DISCUSSION: Many of the proliferating cells were macrophages in the early phase of tendon-bone healing. This suggests that macrophage-derived cells may play a critical role in tendon-bone healing at early time points. Current evidence supports the view that ED1+ macrophages are phagocytic cells that remove cellular debris following injury/trauma. ED1+ macrophages are derived from circulation and migrate into the area of injury. Another subset of macrophages (ED2+) derive from resident cells and are expected to accumulate later. The ED2+ macrophages are felt to have an anabolic role in tendon repair; we are currently localizing ED2+ macrophages in our specimens. Since macrophages have been shown to produce soluble, bioactive cytokines such as IL-1β, IL-6, TNFα, PDGF, and bFGF, which are known to activate and to be mitogenic for fibroblasts, these results suggest that macrophages may play a prominent role in the initiation and regulation of healing of a tendon graft in a bone tunnel. We are continuing to use this model to determine the role of other cells types in the healing process and to examine the fate of the cells in the transplanted tendon using fluorescent in-situ hybridization to identify Y-chromosome containing cells. These results should provide further insight into the process of tendon-bone healing.

REFERENCES:
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Fig 1 (left): Anti-PCNA immunostaining. PCNA+ cells are immunostained with brown color. Fig 2 (right): Double immunostaining technique (brown: anti-PCNA+ cells, blue: ED1+ cells). Bar=20μm

Fig 3 (left) shows microscopic finding of the bone-tendon interface immunostained by ED1 at 4 days after surgery. Fig 4 (right) shows the same area at 14 days after surgery. ED1+ cells (macrophages) were proliferating from the interface (left side of each figure) to the inner tendon (right side of each figure). Bar=100μm.

![Cells in the inner 1/2 tendon](chart)

Table: Cells in the inner 1/2 tendon

<table>
<thead>
<tr>
<th>Days</th>
<th>Total cell</th>
<th>PCNA</th>
<th>ED1</th>
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</tr>
<tr>
<td>28</td>
<td>12</td>
<td>6</td>
<td>6</td>
</tr>
</tbody>
</table>

NS: Not Significant

Fig 5. Cell populations in the inner tendon. Approximately half of the cells that repopulated the tendon graft were PCNA/ED1+ at 14 and 21 days after surgery.