Histological analysis on the tendon-to-bone healing utilizing bone marrow-derived MSCs in ACL reconstruction model without a bone tunnel

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Introduction
To obtain a successful outcome ACL reconstruction, a free tendon graft requires to be securely and biologically anchored to the bone. However, it is well known that the tendon-bone interface is initially mechanical weak point after the operation. Furthermore, the histological structure at the tendon-bone interface is totally different from the highly specialized normal ligament-bone insertion[1].

Previous studies have shown that the graft tendon is initially anchored via fibrous tissue within the bone tunnel wall, and that finally, the integration via chondroid tissue was seen at part of the joint-aperture site[2]. Additionally, myxoid degeneration occurs at part of the intra bone tunnel graft after integration at the joint-aperture site, due to stress shielding. These facts suggest that anchoring within the bone tunnel would no longer be required once integration at the joint-aperture site has been established. In the present study, therefore, to verify whether a structure identical to the normal ligament-bone insertion could be regenerated at the tendon-bone interface without a bone tunnel, we designed an original ACL reconstruction model without a tibial bone tunnel and investigated the histological changes occurring between the bottom of the grafted tendon and the tibial bone. Moreover, to enhance the anchoring process in this model, we transplanted bone marrow-derived MSCs (bMSCs), which are pluripotent cells capable of differentiating into multiple mesenchymal tissues, between the grafted tendon and the bone.

Our first hypothesis was that the grafted tendon would be anchored via chondroid tissue at part of the joint-aperture site even if a bone tunnel was not created. Second hypothesis was that the application of bMSCs at tendon-bone interface would yield results histologically superior to those for controls in ACL reconstruction model without a tibial bone tunnel.

Material and Methods

Experimental Design
14 skeletally mature Japanese white rabbits were used. Ethical approval was obtained from Animal Care Center of Kurume University. Autologous bMSCs were harvested from the bone marrow 2 to 4 weeks before surgery. For bilateral ACL reconstruction in our originally designed model, bMSCs in collagen sponge or fibrin sealant carrier were transplanted between the grafted tendon and the bone in the experimental limb, whereas the control limb received the carrier only. The animals were euthanized at 4 and 8 weeks after the operation, and 7 rabbits at each time point were used for histological analysis.

MSC preparation
The rabbits were anesthetized, and 5 to 10 ml of bone marrow was aspirated from the posterior iliac crest. The anti-coagulated bone marrow was diluted with Hank’s Balanced Salt Solution. The nucleated cell layer was obtained by Ficoll-Paque density gradient centrifugation. The nucleated cells were resuspended in STK-1 containing penicillin 100U/mL and streptomycin 100 U/mL. The cells were plated out at a density of 2.0x10^5/cm^2 in a 6-well plate and grown at 37°C, 5% CO2 in a humidified tissue-culture incubator. The medium was changed every 2 days. After 10 days, the cells were transferred to 6-well plates with STK-2. About 5 to 15 million cells were obtained for use in each model. Survival protocol
Bilateral originally designed ACL reconstruction was performed. A midline incision was made, and the semitendinosus tendon was harvested. The graft was folded in half, and a suture was passed through each end of the graft. After a medial parapatellar arthroscopy was made, the native ACL was excised. Using a 4-mm drill bit, a 4x2-mm bone gutter was created at the tibial attachment site of the ACL, and a bone tunnel (diameter 2mm) was made in the distal femur. In the experimental group, after the graft had been passed through the bone tunnel in the femur, the collagen sponge or fibrin sealant with bMSCs was transplanted between the tibial bone gutter and the graft under slight tensioning. The control group received a transplant of the carrier only. Both ends of the graft suture were secured with buttons. The wound was then closed in layers. The animals were allowed to move freely in their cages after the operation.

Histological and statistical analysis
After removal of the graft-tibia complex, the specimens were fixed in 10% buffered formalin. After decalcification, the samples were embedded in paraffin blocks and cut into 5-μm thick sections longitudinal to the graft. The slides were stained using HE, Safranin-O (SaF-O), and immunohistochemically for collagens type I, II, and III. The tendon-bone interface in each specimen was divided into the anterior and posterior interface, and histological comparison between bMSCs and control group was carried out. Differences in the frequencies of chondroid cell layer formation in each group were cross-tabulated using the χ2-squared test, at a significance value of p<0.05.

Results
At 4 weeks, poorly organized fibrovascular tissue consisted of type III collagen was evident between the grafted tendon and the bone. In the bMSCs group, a chondroid cell layer was formed with Safranin-O and type II collagen was observed at the posterior interface in two of seven specimens, compared with only one of seven specimens in the control group. No chondroid cell layer was observed at the anterior interface in either group. At 8 weeks, the interface was less cellular and had become progressively organized comparison with 4 weeks. In the bMSCs group, a chondroid cell layer was more obvious at the posterior interface in five of seven specimens compared with 4 weeks. In addition, a chondroid cell layer was observed in both the anterior and posterior interfaces in only one specimen[4]. In the control group[4], two of seven specimens showed a chondroid cell layer at the posterior interface, but no such layer was evident at the anterior interface in any of the specimens. A summary of the histological analysis is shown in Table1.

Table1 Chondroid cell layer formation
<table>
<thead>
<tr>
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<th>Anterior</th>
<th>Posterior</th>
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<tr>
<td>bMSCs group</td>
<td>0/7</td>
<td>2/7</td>
<td>1/7</td>
<td>5/7*</td>
</tr>
<tr>
<td>Control group</td>
<td>0/7</td>
<td>1/7</td>
<td>0/7</td>
<td>2/7</td>
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*p<0.05 compared with the anterior interface at 8 weeks in bMSCs group

Discussion
Even in our present ACL reconstruction model without a tibial bone tunnel, integration via chondroid tissue was seen at part of the joint-aperture site. Our first hypothesis was acknowledged. Thus, the bone tunnel was not essential factor as viewed in light of this study. On the other hand, there were no appreciable differences between the groups, and integration between the grafted tendon and the bone was more markedly localized at the posterior interface than at the anterior interface. Thus our second hypothesis was disproved, at least under the specific conditions of this study. However, although only 1 case, an active chondroid cell layer was observed between the grafted tendon and the bone throughout in bMSCs group. Further studies are needed to determine the potential utility of bMSCs application.

Significance
In ACL reconstruction, to enhance the tendon-bone integration without a bone tunnel would lead to save the graft length and avoid the bone tunnel complications (ex. bone-tunnel enlargement after surgery etc.)

References