Acceleration of Tendon-Bone Healing of Anterior Cruciate Ligament Graft Using Autologous Ruptured Tissue

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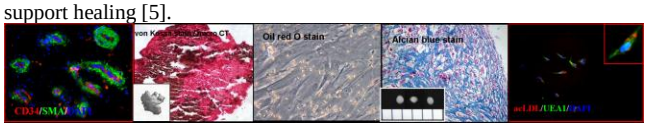
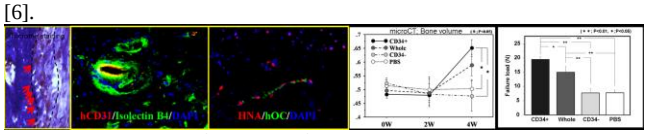
Introduction

Whereas most surgical procedures for anterior cruciate ligament (ACL) reconstruction require healing of tendon grafts in a surgically created bone tunnel, the attachment between the tendon and the bone is the weakest region in the early post transplantation period. Secure fixation of the tendon graft to the bone is a significant factor in allowing earlier and more aggressive rehabilitation and earlier return to sports and work. To this end, tissue engineering using stem cells have recently focused on their potential for early healing and regeneration of tendon bone integration [1]. Although there exist some reports showing the existence of mesenchymal stem cell-like cells in human ACL tissues [2, 3], their origin and characteristics still remain unclear.

Based on the reports showing that blood vessels is a richer supply of stem/progenitor cells with a characteristic of expression of CD34 surface maker [4], we proved that human CD34-expressing vascular cells which had a stem cell characteristics with a potential for high proliferation and multi-lineage differentiation recruited to the ruptured site of ACL to support healing [5].



In addition, we demonstrated that human ACL derived CD34+ cells contributed to tendon-bone healing in an immunodeficient rat model of ACL reconstruction via angiogenesis/vasculogenesis and osteogenesis [6].



Considering clinical feasibility of CD34+ cell transplantation, second step arthroscopic surgery is not avoidable due to the procedure including cell isolation and cell culture. In the previous study, we demonstrated that ACL ruptured tissue contained abundant CD34+ cells (44.3%) compared with intact ACL tissue (8.3%) [5] and that ACL derived non-selected cells as well as CD34+ cells contributed to tendon-bone healing in a rat model of ACL reconstruction [8]. Therefore, using dog ACL reconstruction model as an autologous transplantation model using large animal, we here performed pre-clinical experiments to prove a reasonable hypothesis that ACL ruptured tissue might contribute to tendon-bone healing and regeneration.

Materials and Methods

Animal model of ACL reconstruction: This animal experiment was approved by the Institutional Animal Care and Use Committee of Kobe University. Twenty healthy adult beagle dogs were divided into 2 groups: right knee a tissue-treated group and left knee a control group. ACL was resected 2 days before ACL reconstruction. Each animal underwent bilateral ACL reconstruction using the flexor digitorum superficialis tendon as a graft. In the tissue-treated group, ACL ruptured tissue was sutured to the tibial side of the graft. In the control group, ACL ruptured tissue was discarded. *Histological analysis*: The samples were sectioned parallel to the longitudinal axis of the tibial tunnel. For conventional light microscopy, sections were cut at 5 µm and stained with hematoxylin and eosin (H&E) and Movat Pentachrome (n = 5 in each group at week 2 and 4). *Bone tunnel evaluation by micro CT:* Assessment by computed tomography was previously described [10]. The proximal end of the tibia was cleanly removed from the PMMA resin after biomechanical testing. The areas of a vertical plane of the axis of the bone tunnel at a 10 mm depth from the tibial joint surface were also estimated using computed tomography (CT) (Figure 2A). The areas were measured an average of 3 times with image-J soft ware (n = 5 in each group at week 4). *Biomechanical assessment:* The femur-ACL graft-tibia complex (n = 5 in each group at week 4), with a femur length of 45 mm and a tibia length of 60 mm, was harvested from each knee immediately after sacrifice and frozen at –30°C until testing. All soft tissue except for the ACL graft was resected by sharp dissection, and the tibial postscrew was also removed; the screw fixing the graft to the femur remained in place. All mechanical testing was conducted using a tensile sensor (AG-I SHIMAZU Co, Kyoto, Japan). Immediately after preconditioning, the ultimate load to failure was recorded in uniaxial tension at 20 mm per minute. The load-deformation curve was recorded, from which the ultimate load to failure and the stiffness were measured. *Morphometric evaluation of capillary density and osteoblast density:* Immunofluorescent staining with isolectin B4-FITC conjugate (Vector Laboratories, Inc, Burlingame, CA), an endothelial cell marker, regenerated capillaries and/or neovascularity were visualized and recognized with fluorescence under fluoroscopy at week 2 (n=5 in each group). Immunohistochemical staining was performed with the mouse anti-osteocalcin monoclonal antibody (Thermo Scientific), a osteoblast marker, regenerated osteoblasts and/or enhanced osteogenesis were visualized and recognized with fluorescence under fluoroscopy at week 2 (n=5 in each group).

Results:

Histological Assessment: Early healing inducing endochondral ossification like integration (blue color representation in the staining of Movat Petacrome) in the tissue-treated group was observed, while only reparative tissue was found in the control group. In the granulation tissue at week 4, endochondral ossification like integration was still found in the control group (Fig. 1). *Bone tunnel evaluation by micro CT:* The areas of bone tunnels in the tissue treated group were smaller than in the control group at week 4 (tissue, 19.0 ± 4.4; control, 42.6 ± 4.7 mm^2, $P < .05$ at week 4, n = 5), with a significant difference (Fig. 2). *Biomechanical assessment:* A significantly higher tendon ultimate load at failure was found in the tissue-treated group than the control group at 4 weeks. The mean ± SD on the tensile test was 30.5 ± 10.3 N for the control group at 4 weeks and 66.4 ± 10.1 N for the tissue-treated group at 4 weeks ($P < .05$) (Fig. 3). *Morphometric evaluation of capillary density and osteoblast density:* Capillary density was significantly greater in the tissue-treated group compared with the control group (tissue, 153.6±15.0; control, 103.5±9.9/mm^2, P<0.05). Osteoblast density was significantly greater in the tissue-treated group compared with the control group (tissue, 67.5±5.2; control, 40.5±5.6/mm^2, P<0.05) (Fig. 4).

Figure 1

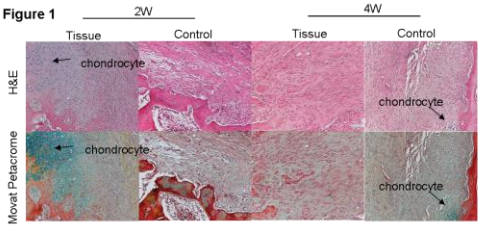


Figure 2

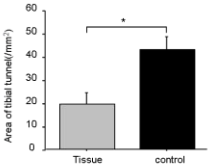
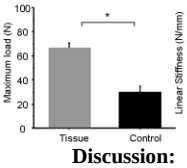


Figure 3



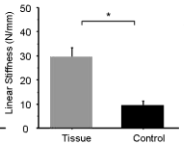
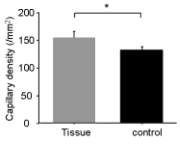
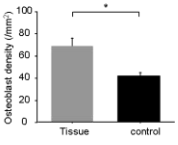


Figure 4





Discussion:

Based on the series of the present study, one-step arthroscopic surgery with ACL-ruptured tissue with easy and surgeons' friendly clinical setting will be possible to be a new strategy for ACL reconstruction surgery.

Significance:

Transplantation of ACL-ruptured tissue containing abundant vascular stem cells contributed to early tendon-bone healing in a canine model of ACL reconstruction.

References:

[1] Petrigliano FA, et al. *Arthroscopy* 22:441-51, 2006. [2] Lee IC, et al. *J Biomed Mater Res A* 83: 241-8, 2007. [3] Lee SY, et al. *J Cell Physiol* 210 : 561-6, 2007. [4] Zheng B, et al. *Nat Biotechnol* 25:1025-34, 2007. [5] Matsumoto T, et al. *Stem Cells Dev* 2011 [Epub ahead of print]. [6] Mifune Y, et al. *Cell Transplant* 2011 [Epub ahead of print].