INTRODUCTION: Tendon and ligament injuries frequently occur in daily activities. It is well known that most cases with these injuries heal naturally in a living body. The process of tendon and ligament healing is a complex one. This process is considered to be separated into four phases, including hemorrhage, inflammation, proliferation, and remodeling or maturation. In the inflammatory phase, various kinds of cells, including bone marrow stromal cells (BMSCs), enter the wounded area. These cells themselves and cellular interactions play a crucial role in the healing process. Especially, BMSCs predominantly contribute to the process of tendon and ligament healing. Regarding the role of BMSCs, there are at least two possibilities in this healing process. One is that BMSCs themselves differentiate into fibroblasts at the injury site. The other is a regulatory effect of BMSCs on enhancing the biological behavior of fibroblasts via cell-cell contact or soluble factors. Herein, little attention has been given to the interactions between tendon or ligament fibroblasts and BMSCs. Much about the role of these interactions remains to be understood. Although our previous study showed that BMSCs have feeder effects on the proliferation, the ability of migration, and the cell adhesivity of rat tendon fibroblasts in an in vitro culture system, the mechanism of these effects remained unclear. The hypothesis of this study was that the feeder effects of BMSCs on fibroblasts would be via some soluble factors. To test this hypothesis, we examined the protein, significantly affecting the fibroblast behaviors in the coculture system, with methods of two-dimensional (2D) electrophoresis and mass spectrometry (MS). The final goal of this study was to identify the proteins that act as soluble factors for affecting the fibroblast behaviors.

METHODS: 1. Membrane-separated coculture. Fibroblasts from WKAH rats (1 × 10⁴ cells) were seeded in BD Falcon™ Multiwell Insert Systems with a micro-porous membrane with feeder cells of BMSCs from WKAH rats (5 × 10⁵ cells, coculture group) or fibroblasts (5 × 10⁵ cells, control group). To test the proliferation of the cocultured fibroblasts, the number of fibroblasts was counted at day 3, 7, 10, and 14 after seeding in each experimental group. (n = 10).

2. 2D electrophoresis and MS analysis for protein identification. To detect some proteins as soluble factors concerning feeder effects, a comparative 2D electrophoresis and MS analysis using the conditioned medium from the coculture and the control groups were performed at Hitachi Science Systems Ltd. (Ibaraki, Japan). The 2D SDS-PAGE was performed on gradient gel (8-16%, 17 cm) by using Protein II xi cell. The spots were visualized by silver staining using a SilverQuest Silver Staining Kit. The 2D images were analyzed and compared by using PDQuest and optical confirmation. The resulting peptide samples were analyzed by time-of-flight MS after being spotted on a MALDI plate and co-crystallized with 2, 5-dihydroxybenzoic acid (DHB). Mass spectra were recorded on a MALDI-TOF instrument (oMALDI-Qq-TOF MS / MS).

3. Cell proliferation assay for the effects of detected protein on fibroblast proliferation. Cell proliferation assay was performed to confirm the effect of the protein detected in the 2D electrophoresis. In brief, fibroblasts were seeded on 96-well plates at 3 × 10³ cells/well with increasing concentration of the detected protein. After 6, 12, 24, 48, and 72 hours incubation, cell number was quantified with WST-8 (n = 5).

RESULTS: 1. Membrane-separated coculture study. The number of fibroblasts significantly increased with time in the coculture group compared to the control (Fig. 1).

2. 2D electrophoresis and MS analysis for protein identification. Figure 2 shows the position of the proteins included in the conditioned medium of the coculture group (A) and the control group (B) on the 2D gels after 2D SDS-PAGE and silver staining.

DISCUSSION: The results obtained here suggest that BMSCs have feeder effects on the proliferation and that plasminogen, as a significantly detected protein in the control medium, has inhibitory effects on the proliferation of cocultured fibroblasts. Although plasminogen itself is thought to be an inactive protein, it has been recognized that angiotatin (kringe 1-4) and kringe 5, the enzymatic cleavages of plasminogen, have potential as an endogenous inhibitor of some types of cells. The mechanism of the inhibitory effect of these proteins is considered to induce apoptosis to target cells. On the other hand, it has also been reported that ligament fibroblasts express plasminogen activators, an enzyme capable of proteolyzing matrix molecules, and plasminogen activator inhibitors and that these enzymes contribute to a more anabolic environment in case of ligament healing. There is a possibility that these enzymes alter the plasminogen activity and capability of influencing fibroblast behavior in this study. This study leads to the conclusion that the feeder effects of BMSCs on tendon fibroblasts are due to the inhibition of plasminogen or its cleavage activity in the culture medium.

REFERENCES:

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