Culture Condition Optimization of Human Anterior Cruciate Ligament- and Bone Marrow-Derived Stem Cells for Ligament Tissue Engineering

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INTRODUCTION:
Tissue engineering with stem cells is a fascinating approach for treating anterior cruciate ligament (ACL) injuries. In our previous study, stem cells isolated from human anterior cruciate ligament were shown to possess extensive proliferation and differentiation abilities under the treatment of specific growth factors. However, the optimal culture conditions and the addition of growth factor fetal bovine serum (FBS) in the in vitro culture system are yet to be determined. In this study, we compared the effects of different culture media with the combination of different growth factors, namely basic fibroblast growth factor (bFGF) and transforming growth factor-β1 (TGF-β1), on proliferation and differentiation of ligament-derived stem cells (LSCs) and bone marrow mesenchymal stem cells (BMSCs).

METHODS:
Isolation of BMSCs and LSCs: Human LSCs and BMSCs were isolated from the same donor receiving arthroscopic procedures for ACL ruptures. The cells isolated were plated as passage 1 with MesenPro (MesenPro RS™ with 2% selected FBS, Invitrogen, Carlsbad, CA, USA) or α-MEM with 10% FBS. The immuno-phenotypes and multi-lineage differentiation abilities of these cells were confirmed. Population doublings of cells: Cells were re-plated in a culture medium of MesenPro or α-MEM with 10% FBS and serially passaged. We calculated the population doubling (PD) for each passage using the formula PD = log[N1/N0]. Effects of growth factors on cell density: Four thousand LSCs and BMSCs were placed in each well of 6-well plates and cultured with growth medium (MesenPro or α-MEM with 10% FBS). The experimental group consisted of growth medium supplemented with 10 ng/mL bFGF or 10 ng/mL TGF-β1. The control group was growth factor free. The number of cells was determined on days 14 by trypan blue exclusion. Quantification of collagen and total ECM proteins: Collagen and proteins were quantified by colorimetric analyses using the selective binding of Sirius Red F3BA to collagen and Fast Green FCF to non-collagen protein. After two weeks of culture, cells in the culture plates were incubated with saturated picric acid solution that contained Sirius Red F3BA and Fast Green FCF. The eluted color was read using a spectrophotometer. Real time RT-PCR analysis: Total RNA from the cells in each group was extracted using a commercially available kit (S.N.A.P.; Invitrogen), according to the manufacturer’s instructions. Quantitative PCR was conducted on a Roche LightCycler® 480 (Roche) real time PCR system. Statistical analysis: Statistically significant differences between groups were analyzed by ANOVA, followed by the Tukey post-hoc test (SPSS version 11.5). A p value of less than 0.05 was considered statistically significant.

RESULTS SECTION:
MesenPro and bFGF increased cell proliferation: The highest cell number was noted in LSCs and BMSCs in MesenPro with bFGF, followed by α-MEM with bFGF (Fig. 1). Shorter population doubling time with MesenPro: The population doubling (PD) time was estimated to be 70.1 and 57.9 hours for BMSCs with α-MEM and MesenPro, respectively, and 74.6 and 58.7 hours for LSCs with α-MEM and MesenPro, respectively (Fig. 2a, 2b). MesenPro with TGF-β1 increased collagen and total ECM protein production: Strong positive staining was noted in the TGF-β1 group, followed by bFGF treatment and the control group (Fig. 3a, 3b). Under spectrophotometric quantification, highest collagen and non-collagen ECM protein production per cell was noted in the TGF-β1 treatment group followed by the control group, and was lowest in the bFGF group of LSCs and BMSCs after treatment for two weeks (Fig. 3c). bFGF and TGF-β1 had opposite effects on mRNA expression of type I collagen, and type III collagen, while all major tendinous matrix genes expression were increased with MesenPro but not α-MEM: The mRNA expression of type I collagen was significantly increased in LSCs and BMSCs with the treatment of TGF-β1 with MesenPro (Fig. 4a). For LSCs, type III collagen mRNA expression was significantly increased with MesenPro, and TGF-β1 with MesenPro. Type III collagen expression was increased in BMSCs treated with MesenPro with TGF-β1, and MesenPro (Fig. 4b). A significantly higher α-smooth muscle actin expression was noted in LSCs and BMSCs with MesenPro and TGF-β1 treatment (Fig. 4c). The mRNA expression of tenascin-c in LSCs was increased with the treatment of MesenPro with TGF-β1. Tenascin-c expression was significantly higher in MesenPro with TGF-β1 treatment of BMSCs (Fig. 4d).

DISCUSSION:
In this study, with α-MEM and 10% FBS in addition to bFGF, rapid proliferation of stem cells can be achieved, while the commercially available medium, MesenPro, is able to promote differentiation of both stem cells toward ligament fibroblasts. Differentiation can be further increased by TGF-β1. With the increasing understanding of the effect of different culture media and growth factors, manipulation of stem cells toward the desired direction for ligament tissue engineering can be achieved.

SIGNIFICANCE: this study successfully confirms that the effect of different culture medium and growth factors on the proliferation and differentiation of stem cells from ACL and bone marrow.

Abbreviations in figures:
LSC: ligament stem cells
BMSC: bone marrow stem cells
M: MesenPro
*: p<0.05
**: p<0.01
αMEM (control): αMEM with 10% FBS.

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