CHARACTERIZATION OF CELLS FOR USE IN LIGAMENT TISSUE ENGINEERING

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ABSTRACT

For potential tissue engineered anterior cruciate ligament (ACL), ACL fibroblasts, medical collateral ligament (MCL) fibroblasts (Lin et al, 1999), mesenchymal stem cells (MSCs) and embryonic stem cells all can serve as cell source theoretically. As ethical debate on embryonic stem cells continues (McLaren, 2001), mesenchymal stem cells (MSCs) are attracting more attention. Not only they have the potential to differentiate into a variety of mesenchymal cell types, including myoblasts and fibroblasts (Pittenger et al, 1999), but also they can easily been obtained by simple aspiration of the anterior superior iliac crest and be amplified *in vitro* to large amount (Jiang et al, 2002 and Colter et al, 2000). In current study, collagen assay and proliferation of ACL fibroblasts, MCL fibroblasts and MSCs were compared as possible cell sources of tissue engineered ACL.

METHODS

Harvest and Culture of MSCs, ACL Fibroblasts and MCL Fibroblasts

Under general anesthesia, 2-3ml of bone marrow was aspirated from iliac crest of male NZW rabbit (2.2-2.5kg). After centrifugation and wash, cells were cultured in DMEM (Sigma, Ph 7.4) supplemented with 10% FBS (GIBCO, 10270-106), 10,000U/ml penicillin/10,000μl/ml streptomycin and 2mM L-Glutamine (Gibco). Hemopoietic cells were removed by medium changing.

Immediately after harvested, ACL and MCL were carefully cut into 1mm×1mm indices and digested with 5ml 0.25% collagenase (Gibco) for 6 hours followed by twice DMEM rinse. Then the systems were incubated at 37º with 5% CO_2 until 80% confluence while medium were changed at three days' interval.

Proliferation Assay

200,000 MSCs (Passage 1 or 2) in 5ml of DMEM with same supplement as before were cultured in 25cm^2 flask until 80% confluence. MSCs were trypsined and counted again. Cell doubling times were calculated from formula,

$$TD=t\cdot lg2/ (lgN_t-lgN_0)$$

in which N_0 and N_t meant primary cell number and acquired cell number respectively.

Collagen Assay

50,000 cells were loaded in one well of 24-well plate. After 24hrs, medium was changed with 0.8ml of DMEM supplemented with 5% FBS, 10,000U/ml penicillin/10,000μl/ml streptomycin and 2mM L-Glutamine (Gibco). Collagen assay were performed according to user manual of Sircol collagen assay kit (Biocolor, UK). Briefly, collected supernatant were centrifuged at 1,500rpm for 4 min to drop extracellular matrix, followed by mixing 100μl supernatant with 1ml of Sircol dye for 30 min and centrifuging at 10,000rpm for 5min to drop formed collagen-dye complex. After decanting suspension, droplets were dissolved in 1ml Sircol alkali reagent and vortexed. 100μl acquired solution were read at 540nm.

Immunohistochemistry

Cells on chamber slide (Iwaki) were stained with collagen I, II, III and smooth muscle actin antibodies (Sigma), using Ultravision Detectin system (Lab Vision Corporation).

RESULTS

Proliferation

The cell number of ACL fibroblasts and MCL fibroblasts acquired from digestion were not stable. From 3 month old male NZW rabbits, 200,000-500,000 fibroblasts could be acquired after 10-14 days' culture with current technique. However, after passage, they deteriorated in no time and were hinted by obvious morphological change and decreased proliferation.

After 18-20 days' culture, 1.5-3m MSCs could be acquired with typical fibroblast-like morphology. P1 MSCs kept previous fibroblast-like morphology and doubled in 50 hours. P2 (25 day) and P3 (30days) MSCs stopped to proliferate as whole with obvious morphological changes. MSCs were positively stained with collagen type I, III and a-smooth muscle actin which existed in ACL and MCL fibroblasts.

Collagen assay

P3 MSCs' collagen excretion decreased slightly when compared with P1 and P2 MSCs, while there is no difference of collagen excretion between P1 and P2 MSCs [Figure 1]. ACL fibroblasts and MCL fibroblasts have obviously lower collagen excretion than P1 MSCs [Figure 2].

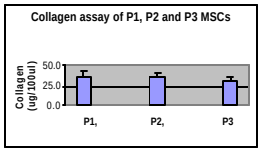

Figure 1. Collagen Assay of P1, P2 and P3 MSCs

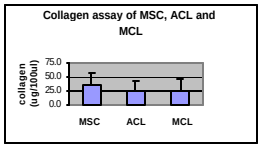

Figure 2. Collagen Assay of MSC, ACL and MCL

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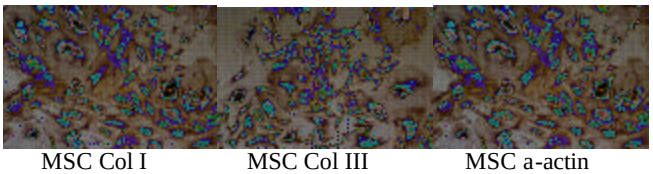
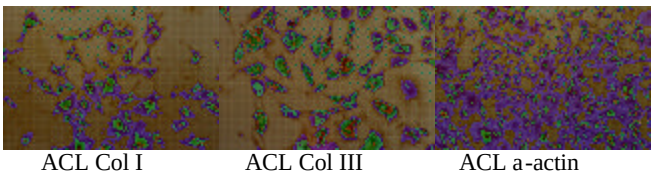
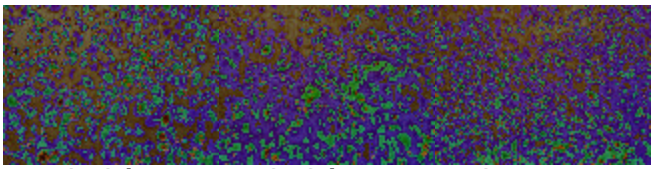


MSC Col I MSC Col III MSC a-actin



ACL Col I ACL Col III ACL a-actin



MCL Col I MCL Col III MCL a-actin

DISCUSSION

Though MSCs have been harvested from periosteum (Nakahara et al, 1991), muscle connective tissues (Nathanson et al, 1980) and adipose tissues (Zuk et al, 2001), bone marrow still keeps being the most accessible source. However, since initial isolation through their adherence to tissue culture surfaces by Friedenstein (Friedenstein et al, 1976), MSCs and their isolation methods have not been well characterized. Several groups of investigators developed protocols to prepare more homogeneous populations (Joyner et al, 1997), but none of these protocols has gained wide acceptance. We used the most popular and simplest protocol to culture MSCs and examined proliferation and collagen excretion after one to three passages. Hopefully our results could guide the further usage of MSCs in tissue engineering field. Contrary to the report that subpopulatin of MSCs could be amplified about 10^9-fold with defined conditions (Jiang et al, 2002), our results showed that MSCs as a whole could not proliferate well after passage 2 (25days) with normal medium. In the meantime, the advantage MSCs have over ACL fibroblasts and MCL fibroblasts in proliferation and collagen and similar properties in collagen type I, III and a-actin with ACl and MCL fibroblasts could potentially broaden their usage in tissue engineered ligament and add the chance to succeed.

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