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Selection of Cell Source for Ligament Tissue Engineering

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Use of appropriate types of cells could potentially improve the functionality and structure of tissue engineered constructs, but little is known about the optimal cell source for ligament tissue engineering. The object of this study was to determine the optimal cell source for anterior cruciate ligament (ACL) tissue engineering. Fibroblasts isolated from anterior cruciate ligament, medial collateral ligament (MCL), as well as bone marrow mesenchymal stem cells (MSC) were compared using the following parameters: proliferation rate, collagen excretion, expression of collagen type I, II, and III, as well as α-smooth muscle actin. Green fluorescent protein (GFP) transfected MSCs were used to trace their fate in the knee joints. MSC, ACL, and MCL fibroblasts were all highly stained with antibodies for collagen types I and III and α-smooth muscle actin while negatively stained with collagen type II. Proliferation rate and collagen excretion of MSCs were higher than ACL and MCL fibroblasts ($p < 0.05$), and MSCs could survive for at least 6 weeks in knee joints. In summary, MSC is potentially a better cell source than ACL and MCL fibroblasts for anterior cruciate ligament tissue engineering.

Key words: Mesenchymal stem cell; Fibroblast; Anterior cruciate ligament; Tissue engineering

INTRODUCTION

The incidence of anterior cruciate ligament (ACL) injuries has increased with increasing popularity in sport activities over the years. About 200,000 Americans required reconstructive surgery of ligaments in 2002 with total expenditure exceeding $5 billion (47,51). The poor healing capacity of the ACL has led to ACL reconstructions in most of the cases. Autografts have been the most popular and successful surgical replacements for the ACL reconstruction (3), but donor site morbidity and availability are major concerns. The use of allograft avoids donor site morbidity and reduces surgical time, but the decrease in tensile properties during sterilization and preservation as well as risk of inflammatory reaction has been a concern (18). Synthetic grafts are seldom used because of poor long-time results (39). Research on potential tissue engineered ACL has been going on for some time, with the hope of overcoming the present problems.

Though reparative cells could be recruited from host tissue through the specific attachment of tissue engineered scaffolds, seeding cells could further improve the functionality of tissue engineered constructs (12,20). Ligament healing may be accelerated secondary to the cellular interaction between local tissue host cells and donor cells while extracellular matrix was being excreted (23). With lack of knowledge about the exact role of seeding cells, it is understood that they are involved in several processes: 1) to attract reparative and/or progenitor cells through chemotaxis signals, 2) to lay down extracellular matrix, which is important to initiate further recruitment of reparative and/or progenitor cells and incorporate and release of endogenous growth factors (6), 3) to elicit immune response. Ideally, seeding cells could survive, proliferate, and excrete extracellular matrix with the right composition as well as elicit mild immune response. Collagen accounts for more than 80% of the dry weight of a normal ligament, and this is mainly collagen I and III with the ratio of six- to eightfold (21, 35). Alpha smooth muscle actin correlates with cell contraction in vivo and in vitro, which is essential for its functionality (11). So it is necessary to examine the expression of collagen I and III and α-smooth muscle actin, as well as proliferation, total amount of collagen excretion, and survival.

Due to differences in phenotypes and functionalities of different cell types, seeding of such cells will greatly influence the outcome of tissue engineered product. Therefore, it is important to select the appropriate cell

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type for the specific application. Logically, ACL fibroblasts should be the primary choice for potential ACL tissue engineering, especially when they could be easily harvested in diagnostic arthroscopic procedures after ACL rupture. On the other hand, medial collateral ligament (MCL), being external to the knee joint and superficial in anatomy, could be easily harvested partially without damaging its functionality in the long term (54). In previous studies, mesenchymal stem cells (MSCs) have been shown to have the potential to differentiate into a variety of mesenchymal cell phenotypes, including osteoblasts, chondroblasts, myoblasts, and fibroblasts (48). They are present in large quantities in the human body and can also be easily obtained just by a simple aspiration of iliac crests and expanded in vitro to large amounts (13,29). MSCs have been successfully used to promote repair of a number of tissue types, including tendon (5), bone (9), ligament (53), and possibly muscle (55). Another possible candidate is the embryonic stem cell (ESC), which has been shown to differentiate into all kinds of somatic cells (15), but its usage is currently hampered by technical difficulties, long-term outcome uncertainty, and ethical considerations (36). Therefore, ESCs were not included in this study.

Hence, ACL and MCL fibroblasts and MSCs are potential candidates for ligament tissue engineering. However, to our knowledge, there are little published data comparing these three cell types. Previous studies on these cells have been based on different species at different ages and cultured with different protocols, and as such it would be very difficult to compare the results. Well-selected cell type loaded in scaffold is effective in enhancing tissue repair and regeneration and could improve the functionality of tissue engineered ligament. As such, we hypothesize that bone marrow-derived MSC is an appropriate cell type for use in ACL tissue engineering applications. The twofold objective of this study was as follows: initially, to evaluate the rate of cell proliferation and collagen expression of ACL fibroblasts, MCL fibroblasts, and MSC, and subsequently, after identification of the optimal cell source, survivability of seeded cells was examined in the knee joint, particularly as an ACL construct.

MATERIALS AND METHODS

Harvest and Culture of ACL Fibroblasts, MCL Fibroblasts, and MSC

In this study, 12 male New Zealand White (NZW) rabbits weighing between 2.2–2.5 kg were used. The ACL and MCL fibroblasts were harvested from the rabbits under sterile conditions according to the method described by Kobayashi et al. (32) and Nagineni et al. (41). After the femoral and tibial insertions of the ligaments were removed, the synovial sheath and periligamentous tissue were stripped away from each ligament. Immediately following the isolation of the ACL and MCL, each ligament was carefully cut into 1×1-mm sections and digested with 5 ml 0.25% collagenase (Gibco) in a 37°C shaking water bath for 6 h followed by twice DMEM rinse. The isolated ligament cells from the two ACLs of the same rabbit were cultured in one 25-cm^2 flask (Corning) with DMEM (Sigma, pH 7.4) supplemented with 10% FBS (Gibco, 10270-106), 10,000 U/ml penicillin, 10,000 μl/ml streptomycin, and 2 mM L-glutamine (Gibco). Then the cell medium system was incubated at 37°C with 5% CO_2 until 80% confluence. The culture medium was changed at 3-day intervals.

The MSC were isolated by using the short-term adherence to plastic method as described by Friedenstein et al. (17). Briefly, the procedure is as follows: the rabbit was put under general anesthesia and 2 ml of bone marrow was aspirated from the iliac crest and subjected to centrifugation. After centrifugation and wash, the cells were cultured in DMEM (Sigma, ph 7.4) supplemented with 10% FBS (Gibco, 10270-106), 10,000 U/ml penicillin, 10,000 μl/ml streptomycin, and 2 mM L-glutamine (Gibco). The cells were cultured in a 75-cm^2 flask (Corning) at 37°C with 5% CO_2 until 80% confluence. The medium was changed at 3-day intervals. Hemopoietic cells were removed at each change of medium.

Subsequently, the cells were trypsinized with 1 ml of 0.25% trypsin-EDTA (Gibco) and subcultured before being used in the evaluation protocol and follow-up experiments.

Proliferation Assay

Cell proliferation assessment of the three cell types (i.e., ACL fibroblasts, MCL fibroblasts, and MSC) was performed after passage 1, with an initial cell number of 0.2 million of each cell type. The cells were cultured in 5 ml of DMEM medium (with the same supplement as described previously) in a 25-cm^2 flask until 80% confluence. Once reached, the cells were trypsinized and counted again. The cell doubling times were calculated from the following equation: $TD = t \times lg2/lgN_t - lgN_0$, where N_0 and N_t are the primary cell number and acquired cell number, respectively.

Collagen Assay

To carry out the collagen assay, 50,000 cells of each cell type were loaded in one of the wells of a 24-well plate. After 24 h, the medium was changed to 0.8 ml of DMEM supplemented with 5% FBS, 10,000 U/ml penicillin, 10,000 μl/ml streptomycin, and 2 mM L-glutamine (Gibco). Collagen assays were performed strictly according to the Sircol collagen assay kit protocol (Biocolor, UK). Briefly, the collected supernatant from the culture well was centrifuged at 1500 rpm for 4 min to

drop the extracellular matrix (ECM); this was followed by mixing 100 μl supernatant with 1 ml of Sircol dye for 30 min and centrifuging at 10,000 rpm for 5 min to drop the formed collagen–dye complex. After decanting the suspension, droplets were dissolved in 1 ml Sircol alkali reagent and vortexed. Subsequently, 100 μl of the acquired solution was read at 540 nm.

Immunohistochemistry

The immunohistochemistry evaluation was carried out on the three cell types; 10,000 cells of each cell type were cultured on each well of the four-well chamber slide (Iwaki, Japan). The four wells were stained with antibodies of monoclonal anti-collagen type I (Sigma, C2456), monoclonal anti-collagen type II (Ab-3, Neo-Markers), monoclonal anti-collagen type III (63172, Chemical Credential, USA), and monoclonal anti-α-smooth muscle actin (1A4, Sigma, A2547), respectively. The UltraVision Detection system (TP-015-HD, Lab Vision Corporation) was used in this study. Predilution of type I, type II, and type III antibodies was 1:4000 whereas α-smooth muscle actin antibody was diluted 1:400. A brief description of the procedure is as follows. After 1 week of cell culture, the cells were fixed in 2% paraformaldehyde for 10 min. The cells were then incubated with six drops of hydrogen peroxide and blocked for 15 min, followed by Ultra V Block for 5 min. Then the cells were stained with the prepared primary antibody for 3 h, biotinylated goat anti-polyvalent for 10 min, streptavidin peroxidase for 10 min, and finally with the freshly prepared DAB solution for 10 min. The cells were washed before each step with PBS.

Green Fluorescent Protein Transfection of MSC

It was found that MSCs had the best proliferation and collagen excretion rate among the three cell types (detailed results of this is reported below); therefore, they were selected as the donor cells. In order to examine the survivability of the donor cells, the primary MSC were transfected with GFP vector (Clontech PEGF FLUO Vector, 6169) and Calphos™ Mammalian Transfection Kit (K2025, Clontech). The MSCs were incubated with the transfection solution, which also included the plasmid DNA and calcium phosphate. The incubation period lasted for 10 h before any change of medium. After 5 days of cell culture, the transfected MSCs were loaded on the knitted scaffold for ACL reconstruction. However, a small amount of MSCs was set aside and cultured on sterile slide for fluorescence screening with a confocal laser microscope (TCS SP2, Leica, German) at 488 nm.

Fabrication of Scaffold

Knitted scaffolds of two biodegradable materials [poly-L-lactic acid (PLLA) and poly-lactic and glycolic acid (PLGA)] were used in this study. The scaffold was knitted out of two PLLA yarns (multifilament, nonbraided, 30 filaments, each filament between 15 and 20 μm in diameter, denier, 83, Scaffix International, USA) and one PLGA yarn (the ratio of PLA and PGA in the copolymer was 10:9, 12 filaments in one yarn and each filament between 15 and 35 μm in diameter, denier 50–60, Shanghai Tianqing Biomaterial, China) in a four-needle knitting machine (SK270, Silver Reed, Suzhou Harisa Machinery Co., China). The two ends of each 5-cm-long knitted structure were sealed with heat. To sterilize the scaffolds, they were immersed in 70% alcohol for 30 min after being washed with PBS twice. Subsequently, the scaffolds were immersed in three changes of PBS for 15 min before use.

MSC Survivability Study

Four skeletally mature male NZW rabbits weighting 3.0 kg or above were used. The anesthesia technique used was as follows: 0.3 ml/kg hyponym was administered intramuscularly to tranquilize the animal; this was followed by intravenous 0.3 ml/kg Valium for induction. Then 1% halothane inhalation was used for maintenance. Cephacexin (0.3 ml per animal) was given subcutaneously as antibiotic.

The surgeries were performed under aseptic conditions. The knee joint was exposed using a midline skin incision and lateral parapatellar arthrotomy, and the patella was dislocated medially with the knee extended. With the knee placed in hyperflexion, the normal ACL was removed by sharp dissection at the tibial and femoral attachment sites while the fat pad was left intact. Using a 2-mm-diameter drill bit (B. Brown) and gas-driven drill (K 100 Air Powered Mini Driver, 3M, USA), bone tunnels were made from the anatomic ACL femur attachment site to lateral femoral condyle, as well as from the anatomic ACL tibial attachment site to medial tibia, which is distal and medial to the tibial tubercle.

Each end of the sterile knitted scaffold was sutured with 4-0 Vicryl suture (Ethicon, NJ); this was to facilitate the ease of threading the scaffold through the tibial and femoral bone tunnels. The scaffold was then loaded with 3 million GFP-transfected MSCs and wrapped with pedicle fascia lata (approximately 0.5 cm wide and 5 cm long) to form the construct. This was to keep the transfected MSCs in the construct. The cell-loaded construct was led through the femoral bone tunnel, the intraarticular space, and the tibial bone tunnel via the 4-0 Vicryl suture and the two ends of the construct were sutured to the periosteum with 2-0 Ethibond nonabsorbed suture (Johnson & Johnson, USA). This was done with the knee at 45° of flexion and the cell-loaded construct in slight tension.

Six weeks later the cell-loaded constructs in the knee

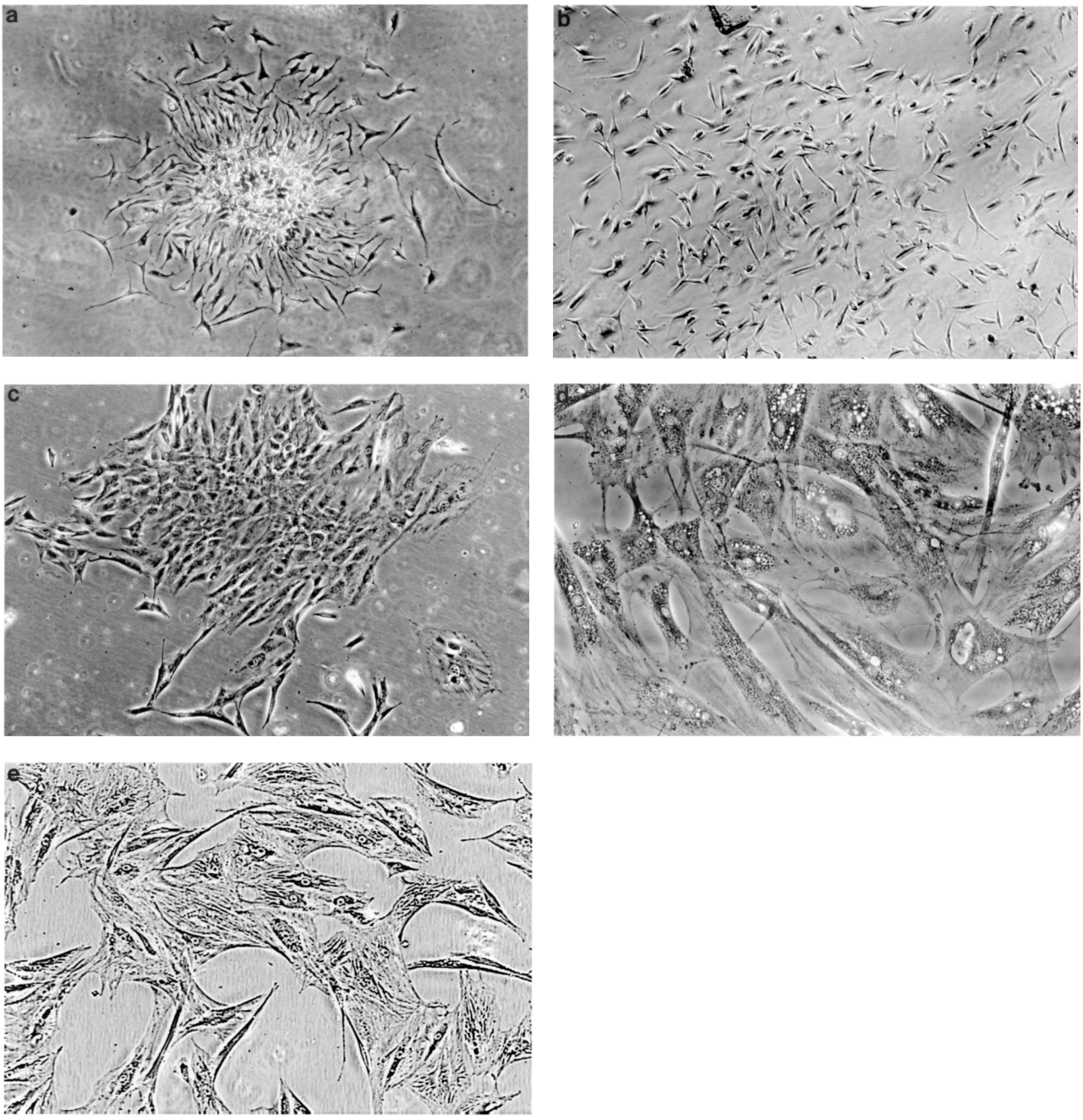


Figure 1. (a) Primary ACL fibroblasts, (b) primary MCL fibroblasts, (c) primary MSC, (d) passage 2 MSC, and (e) passage 3 MSC. Original magnification ×100.

joints were dissected. The retrieved constructs were examined under a confocal laser microscope (TCS SP2, Leica, Germany) at 488 nm.

RESULTS

Cell Proliferation Study

The ACL fibroblasts (Fig. 1a) and MCL fibroblasts (Fig. 1b) acquired from digestion were anchorage dependent and well distributed in flasks, though occasionally showed "clone-like" growth. They grew in monolayers and showed typical fibroblast-like morphology either with spindle shape (bipolar) or stellate (multipolar) pattern. However, cell morphology varied a little between different areas and with the time of the culture. The total numbers of acquired cells were not stable from batch to batch, ranging from 200,000 to 500,000 fibro-

Table 1. Proliferation of MSC ($n = 4$)

	Cell No. at 44 h (in thousands)	No. of Dead Cells at 44 h (in thousands)	Cell No. at 168 h (in thousands)	No. of Dead Cells at 168 h (in thousands)
P1 MSC	362 ± 65.6	—	—	—
P2 MSC	160 ± 15.4	20 ± 5.6	92 ± 23.6	13 ± 2.2
P3 MSC	134 ± 35.5	18 ± 3.1	97 ± 11	15 ± 5

blasts after 10–14 days of culture with the current technique. It was also observed that some of the cells started to show senescence with fatty morphology.

MSCs (1.5–3 million) were acquired with typical colony-forming property and fibroblast-like morphology after 18–20 days of cell culture (Fig. 1c). They were also anchorage dependent and grew in monolayers. In general, they were larger than ACL and MCL fibroblasts, with visible nucleus and nucleolus. After the first passage (P1), it was observed that the MSCs kept the previous fibroblast-like morphology and proliferated well, but after the second passage (P2) at 25 days (Fig. 1d) as well as the third passage (P3) at 30 days (Fig. 1e) the MSCs stopped proliferation while they increased in size and had irregular morphology. MSCs at P3 were even larger than MSCs at P2. The difference in size was obvious when MSCs were trypsinized.

From the equation given above in Materials and Methods, the MSCs at P1 were estimated to double in number at an average of 55.6 ± 14 h ($n = 4$) while the number of dead P1 MSCs was too small to quantify. The total number of MSCs at P2 and P3 dropped after 44 h in current culture conditions, while some of them became detached and died. This was demonstrated by using trypan blue (Sigma, T8154, USA) (Table 1). For the ACL and MCL fibroblasts, after the first passage the cells stopped to proliferate as a whole and started to deteriorate with obvious morphological changes (i.e., increase in size and irregular in shape).

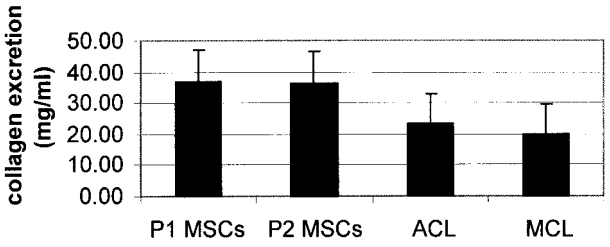


Figure 2. Collagen assays of P1 MSCs, P2 MSCs, ACL fibroblasts, and MCL fibroblasts.

Collagen Assay

MSCs, ACL fibroblasts, and MCL fibroblasts all actively excreted collagen. Collagen excretion of P1 MSCs and P2 MSCs was 37.1 and 36.4 mg/ml, respectively, which was higher than ACL fibroblasts (23.2 mg/ml) and MCL fibroblasts (19.8 mg/ml). There was no significant difference between P1 and P2 MSCs or between ACL fibroblasts and MCL fibroblasts, while collagen excretion of P1 MSCs and P2 MSCs was significantly higher than ACL and MCL fibroblasts (one-way ANOVA, LCD, $p < 0.05$) (Fig. 2, Table 2). Because P2 MSCs had already stopped proliferation, it was not necessary to measure collagen excretion in P3 MSCs.

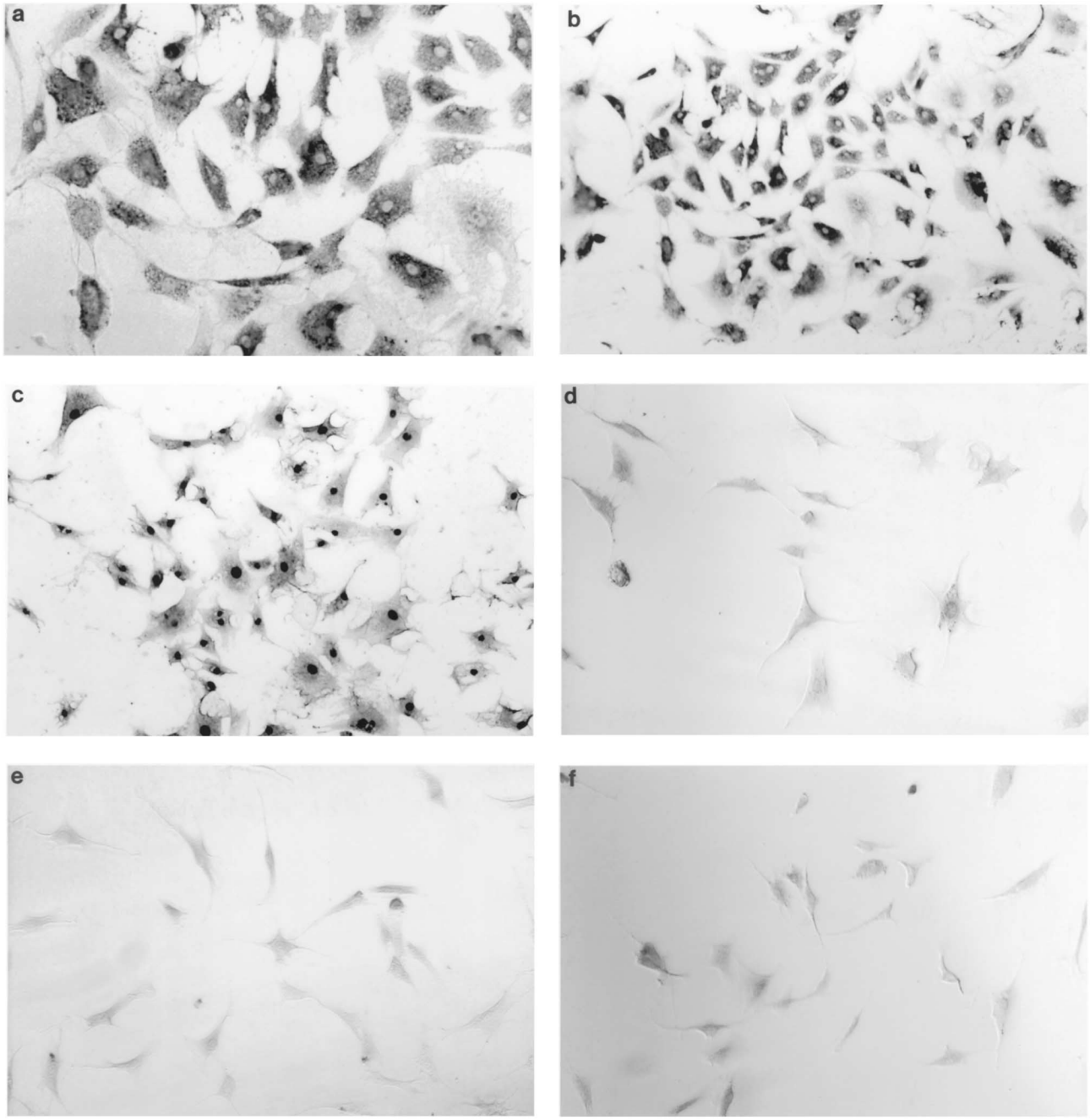
Immunohistochemistry

MSCs were positively stained with collagen type I and type III and α-smooth muscle actin while negatively stained with collagen type II (Fig. 3a–c). In general, the staining density of collagen I was higher than collagen

Table 2. Multiple Comparisons of Collagen Excretion of MSC and ACL and MCL Fibroblasts

					95% Confidence Interval	
I Group	J Group	Mean Difference (I – J)	SE	Sig.	Lower Bound	Upper Bound
P1 MSC	P2 MSC	0.76	1.64	0.65	–2.55	4.06
	ACL	13.94	1.78	0.00	10.37	17.51
	MCL	17.30	2.09	0.00	13.11	21.49
P2 MSC	P1 MSC	–0.76	1.64	0.65	–4.06	2.55
	ACL	13.18	1.94	0.00	9.29	17.08
	MCL	16.54	2.23	0.00	12.07	21.01
ACL	P1 MSC	–13.94	1.78	0.00	–17.51	–10.37
	P2 MSC	–13.18	1.94	0.00	–17.08	–9.29
	MCL	3.36	2.33	0.16	–1.31	8.03
MCL	P1 MSC	–17.30	2.09	0.00	–21.49	–13.11
	P2 MSC	–16.54	2.23	0.00	–21.01	–12.07
	ACL	–3.36	2.33	0.16	–8.03	1.31

Dependent variable: VALUE. The mean difference is significant at the 0.05 level.

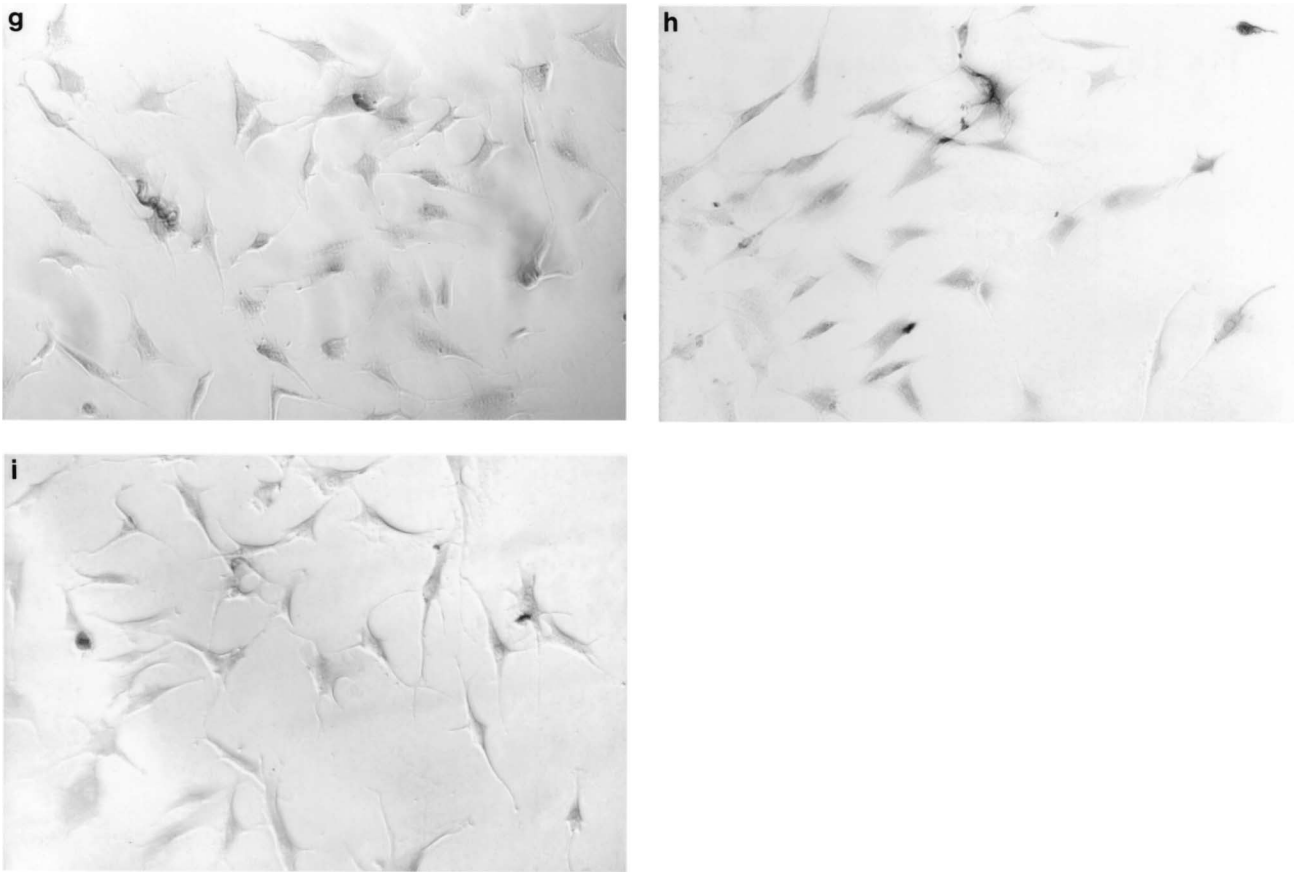


III, while both of them were higher than α-smooth muscle actin. The staining densities of collagen I and III for individual MSCs varied while more than 90% of MSCs were well stained. The staining densities of α-smooth muscle actin for individual MSCs were more homogenous and well distributed in cytoplasma.

Both ACL and MCL fibroblasts were positively stained with collagen I and III and α-smooth muscle actin while negatively stained with collagen II (Fig. 3d–i). The staining densities of collagen I for ACL and MCL fibroblasts were lower than MSCs while there was no difference in density among collagen I and III and α-smooth muscle actin. The stained collagen I and III and α-smooth muscle actin were well distributed in the cytoplasma.

GFP Transfected MSC

Green fluorescence could be detected after 6 weeks in fascia-wrapped constructs while showing individual cell morphology (Fig. 4). They were well distributed in



ABOVE AND FACING PAGE

Figure 3. Staining of MSCs, ACL fibroblasts, and MCL fibroblasts with antibodies of collagen types I and III and α-smooth muscle actin (original magnification 100×). (a) MSCs and collagen type I, (b) MSCs with collagen type III, (c) MSCs with α-actin, (d) ACL cells with collagen type I, (e) ACL cells with collagen type III, (f) ACL cells with α-actin, (g) MCL cells with collagen type I, (h) MCL cells with collagen type III, (i) MCL cells with α-actin.

some areas of explants and surrounded by tissue without fluorescence. In general, green fluorescence only accounted for less than 10% of total areas.

DISCUSSION

Proliferation, protein synthesis, and deposition of extracellular matrix components have been shown to play crucial roles in the repair and remodeling of ligaments (26). In addition, the survival of loading cells is crucial in tissue engineering experiments. It has been found that the rate of proliferation and collagen excretion of MSCs were higher than that of ACL and MCL fibroblasts, while all of them expressed collagen I and III and α-smooth muscle actin, but not collagen II. The loaded MSCs were shown to survive in the knee joints.

Various cells have been used in ligament tissue engineering and many of them have reported the fate of loaded cells in ACL reconstruction with tissue engineering methods (23,28,33,52), but little has been discussed about the functionality of these cells. In previous studies, skin fibroblasts (7) have been identified as a potential candidate for ligament tissue engineering primarily due to their abundance in the body. However, it may not be an appropriate choice for the following reasons. Firstly, harvesting of skin normally results in high morbidity. Secondly, the physiological environment of skin fibroblasts is distinctively different from that of the ACL, which could potentially affect their performance.

MSCs could be harvested from periosteum (42), muscle connective tissues (43), and adipose tissues (56); however, the most accessible and reliable source is the bone marrow. Since Friedenstein et al. (17) reported the initial isolation of bone marrow MSCs through their adherence to tissue culture surfaces, the methods for MSC isolation have not been well developed. Several research groups have developed protocols to prepare more homogeneous populations (24,30,48), including the use of density gradients in an attempt to further separate sub-

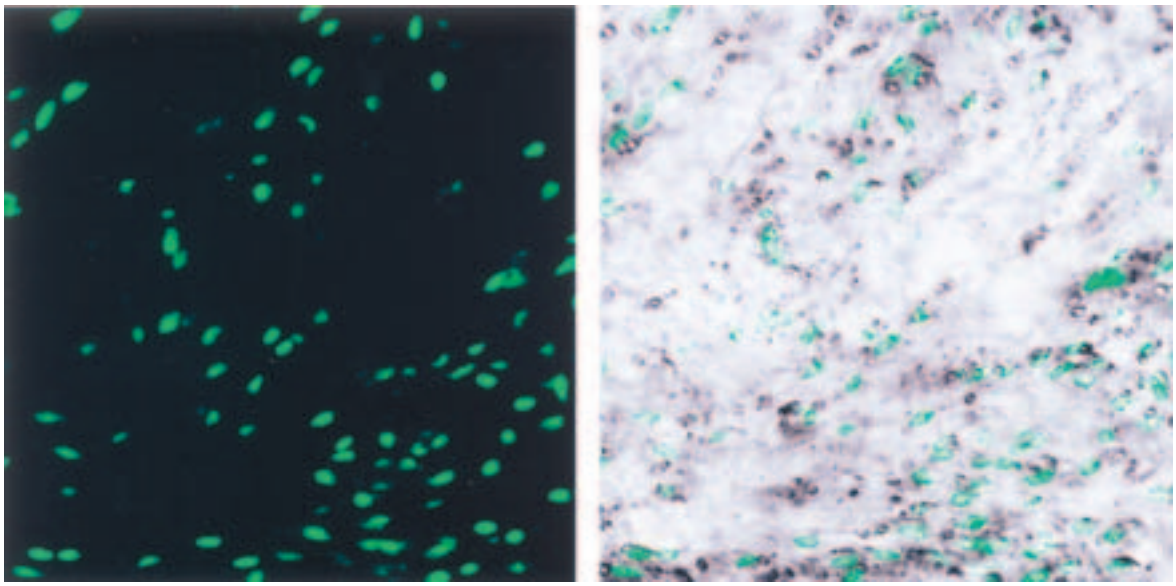


Figure 4. Green fluorescence in 6-week ACL cell-loaded constructs (original magnification 60×).

populations of adherent cells (22), but none of these protocols has gained wide acceptance. In this study, the most common protocol—direct plating (17)—was used to culture the MSC due to the ease of the procedure and wide acceptance in most MSC-related works. Phenotype of the acquired MSCs has been testified by their potential to differentiate into osteo-lineage, chondro-lineage and adipo-lineage (45), as well as the typical well-spread morphology (48).

Previous studies had reported that subpopulations of human MSCs could be amplified by about 20 to 120 population doublings (29,48) and even up to one gigafold (13) in defined conditions before growth arrest and terminal differentiation was observed. However, to our knowledge no similar report has been made in rabbit MSCs. One of the key factors in reduced number of mesenchymal progenitor cells in isolation and culture is age. This had been reported in both human (38) and rabbit (24) studies. Though rabbit MSCs as a whole could not proliferate well after passage 2 (i.e., 25 days) in the current culture conditions, it was not necessary for all subpopulations to stop proliferation, especially when high variability existed in rabbit MSCs (49). The proliferation rate of ACL and MCL fibroblasts was slightly lower than reported in a previous study (41), possibly due to difference in age of rabbits.

Even though serial extraction of collagen has been used to examine the total collagen amount in the extracellular matrix (10), it is also reasonable to measure the collagen excretion ability of cells by analyzing the procollagen (31,44), because procollagen propeptides and mature collagen are synthesized in a ratio of 1:1. The individual collagen polypeptide chains are synthesized on membrane-bound ribosomes and subsequently injected into the lumen of the endoplasmic reticulum (ER) as larger precursor procollagen. After secretion, the propeptides of the procollagen molecules are removed by specific extracellular proteases. This converts the procollagen molecules to truncated but mature collagen molecules, which assemble in the extracellular space to form much larger collagen fibrils (1). It is reasonable for collagen-secreting cells to be well immunostained for collagen I and III as demonstrated in the fibroblasts and MSCs.

Type I collagen is about six to eogjt times far more abundant than type III collagen in ligamentous tissues, while other collagens such as types V, VI, and XII occur in small amounts. However, there was no visible difference in staining density between collagen I and III in the fibroblasts and the MSCs. A probable explanation for this phenomenon could be that the collagen III content was significantly increased during injury (25). The quantitative ratio of collagen I to collagen III is important for the ultimate fibril diameter and functionality (27). In current study, the exact ratio was not determined and was estimated less than its normal ratio. One potential concern is that the functionality of cells in 2D culture is different from 3D. However, we hope that our current study can provide a clue for better understanding.

Fibroblasts play pivotal roles in connective tissue development, physiological remodeling, and wound repair. These cells are likely to comprise heterogeneous populations (34). α-Smooth muscle actin is a functional marker for a fibroblast subtype (i.e., myofibroblast) that rapidly

remodels the extracellular matrix (4). Myofibroblasts containing α-smooth muscle actin also play an important role in the healing process of rabbit medial collateral ligament (16) and maintaining human ACL (40). α-Smooth muscle actin also has been reported in rabbit, canine (11), and murine (46) MSC, but there is no report on rabbit ACL fibroblasts. As in vitro cell culture could increase α-smooth muscle actin expression (8,19), there is still much to study in order to evaluate and quantify the content of α-smooth muscle actin expression in vivo as well as in tissue engineered ligaments.

Previous studies had reported the survivability of donor cells implanted in knee joints. These donor cells can survive for up to 4–6 weeks (7,28,52). However, these studies were performed with different cell sources, animal models, and implantation sites. It would be difficult to make comparisons, as the donor cells may have differences in immunological response, potential blood supply, and fascia protection, etc. Because our results showed that MSCs have the best potential as a cell source for tissue engineering applications, it was necessary to determine the fate of allogeneic MSCs when used in the repair and regeneration of ACL. Two reasons may contribute to the survival of loading MSCs in harsh knee joint environments: protection from fascia cover and mild immune response caused. Though the normal ACL is an intra-articular structure, it is insulated from knee joint fluid by synovium. Usually a simple break in the synovial covering may lead to destruction of the ligament's integrity through exposure to the synovial fluid environment and also by compromising the vascular supply (50). The pedicle fascia used in this study not only provided the essential protective covering for loading MSCs, but also served as a physical barrier to prevent the MSCs from diffusing into the knee joint, as the fibrin glue usually degraded within 1 week (37). Another advantage to using MSCs is a lower immune response. It was reported that neither autologous nor allogeneic MSCs provoked an immune response in vivo, in both local tissue and systemic circulation (2,14). The reason was that MSCs inhibited naive and memory T-cell responses to their cognate antigens (33).

Even though 6 weeks' survival in the knee joints was not long, functionally the loading MSCs should be enough. As the reparative cells and/or progenitor cells continuously grew, the loading MSCs would have gradually been overtaken. The regenerated tissue could have taken up the two main roles of seeding cells, sending chemotaxis signal and laying down extracellular matrix, which are essential in initial stages.

Conclusion

This study has shown that rabbit MSCs have much clearer and distinct advantages over ACL and MCL fibroblasts, with respect to proliferation and collagen excretion. The cultured rabbit MSCs were found to share the same collagen expression types and α-smooth muscle actin as the ACL fibroblasts and MCL fibroblasts. Furthermore, GFP-transfected MSCs were capable of surviving in knee joints for as long as 6 weeks after implantation. Therefore, MSCs were shown to be potentially a better cell source than ACL and MCL fibroblasts for ligament tissue engineering.

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