Expansion of adult equine bone marrow mesenchymal stem cells on fibrinogen-rich protein surfaces derived from blood plasma

Kisiday, J D, Hale, B W, Lee, C M, McIlwraith C W, Frisbie D D
Orthopaedic Research Center, Colorado State University, Fort Collins, CO
john.kisiday@colostate.edu

Introduction: The low density of mesenchymal stem cells (MSCs) in bone marrow requires culture expansion to obtain sufficient numbers for many proposed therapies [1]. Extensive research has focused on conditions for rapid and sustained MSC growth on tissue culture plastic. However, few studies have explored whether other growth substrates could better support MSC expansion. As an alternative to plastic, we evaluated MSC proliferation on plastic coated with a fibrinogen-rich precipitate (FRP) from blood plasma, a material that can be inexpensively obtained and processed using standard clinical and lab equipment and techniques. Post-expansion chondrogenesis was explored as a measure of the effect of expansion conditions on differentiation.

Methods: Bone marrow harvest and MSC isolation: 10 ml of bone marrow was harvested from the iliac crest of 2-5 year old horses. The nucleated cells were seeded into low glucose DMEM + 10% fetal bovine serum (FBS) at 0.25 x 10^7 cells/cm². Adherent MSC colonies were trypsinized after 6-10 days. FRP surfaces: Plasma was obtained from venous blood drawn into CPDA. Plasma proteins were precipitated by mixing 880 μl of 100% ethanol with 5 ml plasma. Precipitated proteins were concentrated by centrifugation, and then resuspended in an equal volume of plasma (200 μl). FRP solution was diluted 1:100 in sterile deionized water and added to flasks at 100 μl/cm². The next day, FRP solution was aspirated, and the flasks were rinsed once with water and dried. For Expt. 1, a subset of FRP surfaces was activated with thrombin (110 NIHU/ml) in 40 mM CaCl₂, diluted 1:20 in water) to convert the bound fibrinogen to fibrin.

Expansion of MSCs: Cultures were conducted in medium consisting of αMEM + 10% FBS that had not been screened for stimulating high rates of MSC growth. In some cultures, the medium was supplemented with 2 ng/ml fibroblast growth factor 2 (FGF2). Expansion cultures were established by seeding at high (10x10⁶ cells/cm²) or low (0.5x10⁶) density. Agarose hydrogel chondrogenesis: MSCs were encapsulated in 2% (w/v) agarose at 10x10⁶ cells/ml and cultured in high glucose DMEM, 1% ITS+, 0.1 μM dexamethasone, 37.5 μg/ml ascorbate-2-phosphate, and 10 ng/ml rhTGFβ-3 [2]. After 15 days, samples were digested in proteinase K, and the total accumulated sulfated glycosaminoglycan (GAG) content was measured via DMMB dye binding assay. Selected samples were evaluated for type II accumulation via immunohistochemistry.

Statistical Analysis: A log-transformed model analysis of variance, with donor animal used as a random effect. Individual comparisons were made using least square means procedure. For each experiment, MSCs were tested from 5 donor horses.

Results: Experiment 1 - MSCs were seeded on FRP, with and without thrombin activation, at high density and cultured for 5 passages in FGF2 medium. Control MSCs cultured on uncoated tissue culture plastic in FGF2 averaged a population doubling (PD) every 24.5 hours. The cumulative PDs on FRP and FRP/Thrombin was greater than uncoated cultures by the first passage (Fig. 1, p < 0.05). By passage 5, growth in FRP and FRP/Thrombin cultures had exceeded uncoated tissue culture plastic by 1.2 and 1.5 PDs, respectively. Cumulative PDs were similar for FRP and FRP/Thrombin surfaces (p = 0.73-0.93). GAG accumulation was similar among surfaces after 2 and 5 passages (Fig. 2, p = 0.13-0.86). For uncoated and FRP cultures, GAG accumulation at passage 5 was 72% of passage 2 (p = 0.05). For FRP/Thrombin, GAG accumulation at passage 5 was 82% of passage two, although this difference was not significant (p = 0.08). Type II collagen immunohistochemistry – Type II collagen was detected in all samples (data not shown). At passage 2 and 5, the intensity of staining was qualitatively similar among culture expansion conditions. The intensity of staining in passage 5 samples was moderately lower than passage 2.

Experiment 2 - MSC expansion was evaluated on FRP, without thrombin activation, at high density in the absence of FGF2. Uncoated cultures, in the presence and absence of FGF2, were maintained at high density as controls. Cumulative PDs in uncoated/FGF2- were less than uncoated/FGF2+ at each passage (Fig. 3, p < 0.05). In FRP/FGF2- cultures, cumulative PDs were greater than uncoated/FGF2- conditions beyond the first passage (p < 0.05). Cumulative PDs in FRP/FGF2- were less than uncoated/FGF2+ beyond the second passage (p < 0.05). By passage 5, FRP/FGF2- cultures had experienced 3.7 more PDs than uncoated/FGF2- cultures but 1.5 fewer than uncoated/FGF2+.

After 2 and 5 passages, GAG accumulation in MSC-seeded agarose was similar among conditions [4, p = 0.46-0.85]. After 5 passages, GAG accumulation in uncoated/FGF2- and FRP/FGF2- cultures decreased to 69% and 67% of passage 2 values, respectively (p < 0.05). In uncoated/FGF2- samples, passage 5 GAG accumulation decreased to 77% of passage 2 values; however, this change was not significant (p = 0.13-0.86). For uncoated and FRP cultures, GAG accumulation decreased to 69% and 67% of passage 2 values, respectively (p < 0.05). Proportion of FGF2+ cells expressed in MSC-seeded agarose was similar among conditions (Fig. 4, p = 0.46-0.85). After 5 passages, GAG accumulation in uncoated/FGF2- and FRP/FGF2- cultures decreased to 69% and 67% of passage 2 values, respectively (p < 0.05). In uncoated/FGF2- samples, passage 5 GAG accumulation decreased to 77% of passage 2 values; however, this change was not significant (p = 0.13-0.86).

Experiment 3 - MSC proliferation on FRP at low density was compared to low and high density cultures on tissue culture surface. All culture medium contained FGF2. On uncoated surfaces, low density cultures experienced 1.2 (day 4) and 2.8 (day 8) more PDs than high density cultures (Fig. 5, p < 0.05). Proliferation on FRP exceeded uncoated low density cultures (0.5 and 1.2 additional PDs on day 4 and 8, respectively, p < 0.05). Proliferation in low-density cultures was extended through day 18, where FRP cultures resulted in 3.9 additional PDs relative to uncoated (p < 0.05). Throughout the proliferation timecourse, uncoated low density cultures produced dense, colony-like patterns of growth, while FRP supported a more homogeneous distribution across the growth surface.

Discussion: The continued advancement of MSC-based regenerative strategies will necessitate clinical expansion techniques that generate a consistent yield of MSCs. Variability among patient populations, such as MSC yield from bone marrow, proliferation rates, and differentiation capacity that can change with age [3,4] may challenge expansion consistency and slow enthusiasm for clinical implementation. Given the ability of FRP surfaces to stimulate MSC proliferation without affecting chondrogenesis, it appears that FRP surfaces may represent a simple autologous method to enhance proliferation of both rapid (FGF+) and slow (FGF-) growing MSCs. Expansion of MSCs in FGF2 at low density appears promising [5], with the greatest cell yield achieved for FRP surfaces, although ongoing studies must determine whether FRP surfaces influence differentiation potential.