Comparison of Human Mesenchymal Stem Cells Derived from Various Spinal Tissues: Superiority of Facet Joint and Interspinous Ligament

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Disclosures: B. Kristjánsson: None. W. Limthongkul: None. S. Honsawek: None.

Introduction: Mesenchymal stem cells (MSCs) capable of osteogenic, chondrogenic, and adipogenic differentiation have been gaining much interest for therapeutic applications [1]. Residing predominantly within the bone marrow and adipose tissue they are also found most connective tissues of the human body where they are involved in the maintenance and regeneration [2]. Lumbar spinal stenosis and ossification of the posterior longitudinal ligament (OPLL) are pathological conditions characterized by the hypertrophy and calcifications of connective tissues within the spinal column. The precise etiologies of these conditions are poorly understood. Compression of the spinal canal often leads to surgery where calcified and hypertrophied structures are removed. Heretofore, few have addressed MSCs roles in these conditions; they have been isolated and described from the ligamentum flavum and posterior longitudinal ligaments. MSCs from OPLL patients have been evinced to exhibit significantly stronger osteogenic potentials compared to MSCs from healthy controls [3]. Accordingly, the objective of this study was to identify whether MSCs could be isolated from ligamentum flavum, interspinous ligaments, and facet joints of lumbar spinal stenosis patients.

Methods: Spinal ligaments samples were harvested from patients during posterior decompression surgery. Cells were isolated from ligamentum flavum, interspinous ligaments, and facet joints via direct tissue explants and grown to confluence in culture medium (α-minimum essential medium supplemented with 10% fetal bovine serum and 200 U/mL penicillin/streptomycin). Clonogenicity was measured by colony forming unit-fibroblast (CFU-F) assay where 10 cells were seeded into each well of 6-well plates, incubated for 14 days and colonies counted afterwards. To measure cell proliferation, cells were seeded at low density (1,250 cells/cm²), incubated for 14 days, counted and population doublings calculated. Expressions of the MSCs surface markers CD29, CD44, CD90, CD105 and the hematopoietic markers CD34 and CD45 were established using flow cytometry. Tri-lineage differentiation was initiated by growing passage 2-3 cells in osteogenic, chondrogenic and adipogenic medium for 21 days. For osteogenic and adipogenic differentiation 1.5x10⁵ cells were seeded in 6-well plates. Osteogenic medium consisted of culture medium supplemented 10 nM dexamethasone, 10 mM β-glycerophosphate, and 50 µg/mL ascorbate-2phosphate. Adipogenic medium consisted of culture medium supplemented with 100 nM dexamethasone, 50 µg/mL indomethacin, and 0.45 mM 3-isobutyl-1-methylxanthine. For chondrogenic differentiation approximately 1.0 x 10⁶ cells were grown in a pellet culture. Chondrogenic medium consisted of culture medium supplemented with
10 nM dexamethasone, 10 ng/mL transforming growth factor-β3, and 6.25 μg/ml insulin-transferrin-selenium (ITS supplement). For histological staining, osteogenic cultures were stained with Alizarin Red, adipogenic cultures were stained with Oil Red O, and chondrogenic pellets were embedded in paraffin, cut into 4 μM thin sections and stained with Toluidine blue. Gene expression the osteogenic, adipogenic and chondrogenic genes was analyzed using reverse transcription-polymerase chain reaction.

**Results:** High-proliferating, spindle-shaped, plastic adherent cells emerged from the planted tissue samples after 1-2 weeks of incubation. CFU-F assay confirmed that all cell lines were clonogenic with up to 32.2% colony-forming efficiency. Cells exhibited high-proliferation and cell proliferation assay evinced that cells could be grown for maximum 5 passages when seeded at low density and reaching up to 36.4 population doublings ([Fig. 1](#)). Flow cytometry revealed cultures to be partly or fully positive for common MSCs markers CD29, CD44, CD90 and CD105, whilst being fully negative for hematopoietic markers CD34 and CD45 ([Fig. 2](#)). All cell lines were able to undergo tri-lineage differentiation. Calcium accumulation in osteogenetic cultures was observed by strong Alizarin Red staining in the induction medium whilst absent in control culture. Lipid droplets were detected in adipogenetic culture by red staining revealed by Oil Red O whilst not seen in the control culture. Extracellular matrix was observed in chondrogenic sections and stained blue with Toluidine blue, whereas no extracellular matrix was observed in the control pellets (Fig. 3). Markers associated with osteogenic, adipogenic, and chondrogenic differentiation were expressed in the induction cultures and not control cultures as detected by reverse transcription-polymerase chain reaction. The rate of alizarin red-positive colonies was higher in facet joint- and interspinous ligament-derived cells. In adipogenesis, number of oil red O-positive colonies was higher in facet joint- and interspinous ligament-derived cells. In studies of chondrogenesis, pellets from facet joint-and interspinous ligament-derived cells were shown to be larger and stained more extensively for cartilage matrix ([Fig. 3](#)).

**Discussion:** This study reveals that cells capable of tri-lineage differentiation are found within the ligamentum falvum, interspinous ligaments, and facet joints. These cells fulfill the criteria for MSCs, capable of forming colonies, showing high-proliferation, tri-lineage differentiation, and they display the markers frequently associated with MSCs [4]. Our findings suggest that there are differences in MSC properties according to tissue source, beyond donor variation. Superiority of facet joint and interspinous ligament could serve as potential sources of MSCs for clinical applications.

**Significance:** The present study demonstrates that MSCs can be found in various spinal tissues. They may be potential alternative sources of MSCs for clinical use in tissue regeneration.
Fig. 1 Cell proliferation assay. MSCs from facet joints (FJ) and interspinous ligaments (IL) proliferated at similar rates whilst MSCs from ligamentum flavum (LF) slowed down at passage 3.
Fig. 2 All cell lineages were positive for CD29, CD44, CD90 and CD105 and negative for CD34 and CD45.
Fig. 3 Histology staining. (A-C) Osteogenic induction and (D-F) control stained with Alizarin Red. (G-I) Adipogenic induction and (J-L) control stained with Oil Red O. (M-O) Chondrogenic induction and (P-R) control stained with Toluidine blue.