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Effects of Hypoxia and TGF-β Exposure during Monolayer Expansion on the Survival and Matrix Producing Capacity of Mesenchymal Stem Cells

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Introduction

Degeneration of the intervertebral discs is implicated as a major cause of lower back pain[1]. There is a need for treatment options that not only alleviate symptoms but also reconstitute native tissue structure and mechanical function within the disc. Over the past several years, application of mesenchymal stem cells (MSCs) for disc regeneration, particularly for the nucleus pulposus (NP), has received considerable attention. Previous studies have shown that MSCs are capable of undergoing differentiation into a NP-like phenotype under certain culture conditions[2-4]; however, a key challenge to successful application of MSCs for NP regeneration is the harsh *in vivo* environment. The NP region of the disk, which is characterized by low nutrition and oxygen tension, both of which may negatively impact the survival and biosynthetic properties of MSCs[5]. The objective of this study was to investigate whether exposing MSCs to hypoxia during monolayer expansion enhances subsequent survival and regenerative potential in the nutrient and oxygen poor NP environment. Furthermore, we investigated whether priming MSCs towards an NP-like phenotype by exposing them to TGF-β3 during monolayer expansion enhances subsequent regenerative potential.

Methods

Cell Isolation and Expansion

Bone marrow-derived MSCs were isolated from 3 juvenile bovine femurs and tibia (<6 months of age), pooled, and expanded to confluence through a single initial passage in monolayer in normoxia (21% O_2) and basal medium (DMEM (4.5 g/L glucose) and 10% FBS). The cells were then passaged and expanded in basal medium in one of four different conditions for 1 week: 1. Normoxia (21% O_2; standard MSC expansion conditions); 2. Normoxia+TGF-β3 (10 ng/mL); 3. Hypoxia (2% O_2); 4. Hypoxia+TGF-β3 (10 ng/mL).

Pellet Culture

After the monolayer expansion protocol described above, cells were passaged and cultured in pellets (250,000 cells/pellet) in a simulated NP-like environment (hypoxia (2% O_2) and chemically defined media with low glucose (1 g/L) DMEM and no growth factors)). After 2 weeks of culture, pellets were harvested and either fixed in formalin and processed for paraffin histology (n = 2) or analyzed for biochemical composition (n = 5). For histology, sections were stained with Alcian blue (glycosaminoglycans, GAG) or picrosirius red (collagen). For analysis of biochemical composition, DNA, GAG, and collagen contents were quantified using the PicoGreen (Thermo Fisher), dimethylmethylene blue, or hydroxyproline assays respectively. DNA was analyzed per pellet, and GAG and collagen were normalized to DNA. Significant differences ($p < 0.05$) between groups were established using 2-way ANOVA with Bonferroni post-hoc tests ($p < 0.05$).

Microarray Analysis

Bovine MSCs were isolated and expanded under the four conditions described above, with cells from 3 different donor animals maintained as distinct biological replicates. Cells were harvested, high quality RNA (RIN > 9) was isolated from each sample, and global gene expression was measured using the WTPlus Bovine Gene Chip (Affymetrix GeneChip system). Gene expression data were normalized using Robust Multi-array Average. Significant differences in gene expression were determined using 3-way mixed model ANOVA ($p < 0.05$; adjusted for false discovery rate).

Results

Pellet Culture

DNA content for pellets with MSCs expanded in hypoxia, both with and without TGF-β3, was significantly higher than for those with MSCs expanded in normoxia, both with and without TGF-β3 (Figure 1A). DNA content was lowest for pellets with MSCs expanded in normoxia with TGF-β3, and highest for pellets with MSCs expanded in hypoxia with TGF-β3. There was no significant effect of monolayer expansion condition on pellet GAG content (normalized to DNA, Figure 1B). Collagen content exhibit the opposite trend to DNA, and was highest

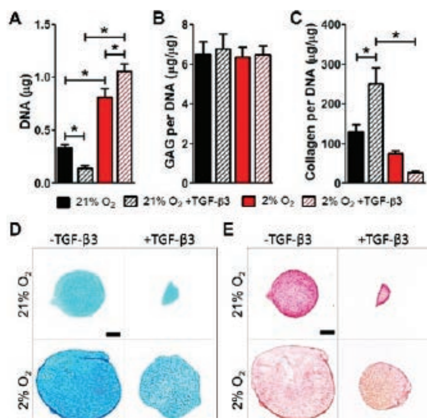

Figure 1. Composition of MSC pellets after monolayer expansion in different oxygen and TGF-β3 conditions. A. DNA content, B. GAG per DNA, and C. Collagen per DNA. D. Alcian blue staining for GAG. E. Picrosirius red staining for collagen. *$p < 0.05$; scale bar = 0.2 mm.

for pellets with MSCs expanded in normoxia with TGF-β3 ($p < 0.05$ vs both normoxia without TGF-β3 and hypoxia with TGF-β3, Figure 1C). Histological results supported these findings (Figs 1D and E), where pellets with MSCs expanded under hypoxia, with and without TGF-β3, were larger than those with MSCs expanded under normoxia, suggesting higher cell numbers.

Microarray Analysis

Principal component analysis (PCA, Figure 2A) indicated significant effects of MSC donor on the global gene expression in response to each expansion condition.The effects of altering oxygen tension alone (without TGF-β3) during monolayer expansion on MSC gene expression were moderate. MSCs expanded under hypoxia exhibited differential expression of genes implicated in the cell stress response (B4GALT56: galactosyltransferase; LPL: lipoprotein lipase; NGF: nerve growth factor; PK: pyruvate kinase) compared to normoxia expanded MSCs (Figure 2B). Exposure to TGF-β3 during monolayer expansion resulted in the greatest effects on global gene expression, irrespective of oxygen tension. In particular, there were significant effects on expression of genes involved in growth and inflammation, including those of the TGF-β, NFκB, and caspase activation pathways (Figure 2C).

Discussion

The results of this study suggest that exposure to hypoxia during monolayer expansion leads to improved survival (higher DNA content) when these cells are subsequently cultured in simulated NP-like conditions with limited oxygen and nutrition. Interestingly, exposure to hypoxia during monolayer expansion had no significant impact on the subsequent matrix (GAG or collagen) producing capacity of MSCs in the absence of TGF-β3. In contrast, exposure to TGF-β3 under normoxic conditions during expansion significantly inhibited subsequent MSC survival and boosted collagen production on a per cell basis with no effect on GAG. This may suggest induction of a post-mitotic and pro-fibrotic phenotype, which may be detrimental to the capacity of MSCs

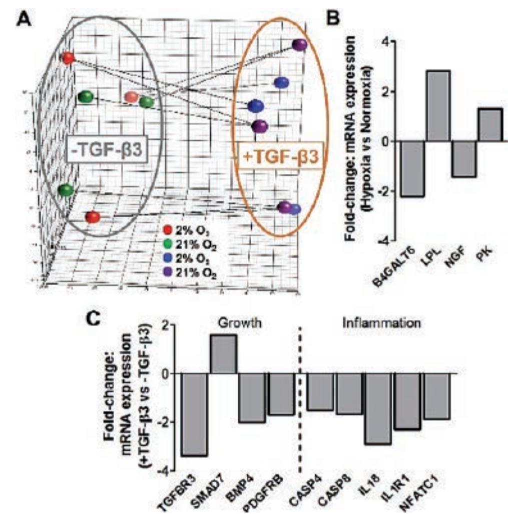

Figure 2. Microarray results. **(A)** Principal component analysis (PCA) plot. Lines connect all samples from a single animal. **(B)** Effects of hypoxia on gene expression in the absence of TGF-β3. **(C)** Effects of TGF-β3 on growth and inflammation pathway gene expression. N = 3; all $p < 0.05$.

to regenerate NP tissue. Microarray results support this view, with TGF-β3 exerting significant effects on signaling pathways that regulate fibrosis and inflammation, which eclipsed any beneficial effects of hypoxia alone. Ongoing work will seek to verify these findings, by determining the type of collagen (I or II) being produced and measuring levels of pro-inflammatory factors in the culture media. Finally, microarray results highlighted the significant effects of donor on the response of MSCs to environmental stimuli, potentially due to variations in age and sex, the impact of which should be considered during future translational studies.

Significance

The results of this study demonstrate that alterations in monolayer expansion environment significantly impact the survival and matrix producing capacity of MSCs and provide a foundation for optimizing the regenerative capacity of these cells in the intervertebral disc.

Acknowledgements

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