Adipose-Derived Stem Cells Retain their Regenerative Potential after Methotrexate Treatment

Olivia S. Beane, Vera C. Fonseca, MS, Eric M. Darling, PhD.
Brown University, Providence, RI, USA.

Disclosures:
O.S. Beane: None. V.C. Fonseca: None. E.M. Darling: None.

Introduction: MTX is a chemotherapeutic agent that targets dihydrofolate reductase (DHFR), a protein involved in DNA biosynthesis. Treatment with MTX leads to inactivity of DHFR, inhibiting DNA production and leading to cell damage. MTX is used to treat many cancers, including acute lymphoblastic leukemia, the most prevalent form of childhood cancer. While chemotherapeutics have increased survival rates of patients, they have also been implicated in long-term side effects including muscle dysfunction, increased marrow fat volume, decreased bone mineral density, growth arrest, osteonecrosis, and fractures over the course of the patient’s lifetime. Recent findings indicate that these side effects are consequences of BMSC impairment, whereby MTX reduces the osteogenic potential, and increases the adipogenic potential, of BMSCs. As such, it is necessary to examine how MTX influences the function of other stem cell types to identify a more beneficial cell source for musculoskeletal regeneration. Resistant stem cells could also potentially prevent tissue damage altogether by assuming the role of BMSCs prior to MTX treatment. Furthermore, determining the mechanism behind stem cell resistance would provide insight for devising preventative treatments for BMSCs exposed to MTX.

The objective of this study was to investigate the ability of human, adipose-derived stem cells (ASCs) to resist MTX damage and compare their response with normal human fibroblasts (NHFs), a non-cancerous somatic cell type. We hypothesized that ASCs would maintain their proliferative abilities after treatment with varying concentrations of MTX, whereas NHFs would senesce at high drug concentrations. We also hypothesized that DHFR expression would be upregulated more in ASCs than NHFs after MTX treatment, indicating a mechanism for their resistance. Lastly, we hypothesized that ASC multilineage differentiation potential would be unaffected by MTX.

Methods: For proliferation studies, ASCs and NHFs were plated in 96 well plates at 2,000 cells/cm² (N = 2 iterations, n = 12 samples). After adhering overnight, cultures were treated with MTX concentrations at or above clinical relevance (0.1 - 50 μM) for 24 hours. Cells were cultured in expansion media for an additional 10 days to assess recovery and proliferation. Cell numbers were quantified on days 6-10 by correlating the fluorescence of Hoechst-stained nuclei with cell number via a spectrophotometer, a methodology established previously in the lab. Western blots were conducted to determine DHFR expression of untreated and MTX-treated ASCs and NHFs (N = 3 iterations, n = 12 replicates). Cells were plated in flasks and expanded until 80% confluence, at which point they were treated with 0 or 2.5 μM MTX for 24 hours. Western blots were run to determine the presence and amount of DHFR, normalized to actin levels. Comparisons were made between bands of untreated and MTX treated samples to determine relative changes in protein expression for ASCs and NHFs.

To investigate the effects of MTX on ASC differentiation potential, cells were plated and treated with 0 or 2.5 μM MTX, as described for proliferation (N=1 iteration, n=4 samples). Cells were expanded for an additional 6 days after treatment and then re-plated for differentiation. ASCs were induced along the adipogenic, osteogenic, and chondrogenic lineages using previously described protocols. Lineage-specific metabolites were quantified by measuring the optical density of eluted stain for lipids (adipogenesis), calcified matrix (osteogenesis), and sulfated glycosaminoglycans (chondrogenesis). All optical densities were subsequently normalized to cell number (adipogenesis and osteogenesis) or DNA amount (chondrogenesis). Two-way ANOVA was conducted to analyze proliferation (MTX concentration and day) and differentiation potential (MTX treatment and differentiation condition). Tukey’s HSD post-hoc test was used to determine statistical significance for individual comparisons (p < 0.05).

Results: ASCs resisted the anti-proliferative effects of MTX, whereas NHFs senesced after treatment. Statistical analysis of cell growth determined no significant reduction in ASC counts at any time points or any concentration of MTX (Fig. 1A). Interestingly, cell counts for ASCs treated with 50 μM MTX were significantly higher than all other ASC populations (p < 0.05). Conversely, at all time points, NHF cell counts were significantly lower than respective controls after treatment with 0.5-50 μM MTX and were only 5-15% of control cell counts on day 10 (p < 0.05). Western blot revealed that increases in DHFR after MTX treatment were 2300% greater in ASCs than NHFs (Fig. 2).

MTX did not impair the differentiation potential of ASCs. Comparable amounts of lipid and calcified matrix production were observed in untreated and MTX treated samples, as determined by histological staining (Fig. 3A). Though MTX slightly enhanced lipid production in induced (11%) and control samples (20%) (Fig. 3 B, p < 0.05), this change was minor. Statistical analysis of osteogenesis and chondrogenesis determined no significant differences existed between untreated and MTX treated samples.
Therefore, ASC multilineage differentiation potential was unaffected by MTX treatment.

**Discussion:** ASC regenerative properties, and hence, therapeutic potential, were not affected by MTX. Proliferation experiments determined that MTX did not inhibit ASC growth, but instead, stimulated proliferation. Conversely, NHF growth was inhibited by MTX treatment, as expected. ASC resistance to MTX is hypothesized to be due to greater upregulation of DHFR. Normally, MTX not bound by DHFR will bind all available protein in the cell, preventing DNA biosynthesis. Greater upregulation of DHFR provides excess amounts, enabling the continuation of necessary bioprocesses. ASC resistance and recovery was further confirmed by showing that 2.5 μM MTX does not impair adipogenesis, osteogenesis, or chondrogenesis. These results are encouraging, as previous studies have determined that as low as 0.1 μM MTX alters BMSC differentiation potential. As such, ASCs may be a viable stem cell source for regeneration or prevention of musculoskeletal deficiencies in cancer patients.

**Significance:** The chemotherapeutic agent methotrexate (MTX) impairs bone marrow stem cell (BMSC) function, leading to long-term musculoskeletal deficiencies for which there are currently no treatment options. We have determined that adipose-derived stem cells (ASCs) are resistant to MTX, thus identifying a potential source to regenerate or prevent bone loss of patients undergoing MTX treatment.

**Acknowledgments:**

Figure 1. Proliferation of ASCs (A) and NHFs (B) after 24 hours treatment with methotrexate. ASCs: adipose-derived stem cells; NHFs: neonatal human foreskin fibroblasts.
Figure 2. Western blots indicated that ASCs upregulate DHFR 2-fold more than NHFs in response to 2.5 μM MTX.