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Disparate Response to Methotrexate in Stem versus Non-Stem Cells

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Abstract

Methotrexate (MTX) is a commonly used chemotherapeutic agent that kills cancer cells by binding dihydrofolate reductase (DHFR) as a competitive inhibitor. Due to its non-selectivity, MTX also impairs normal (non-cancerous) cell function and causes long-term damage to healthy tissue. These consequences have been investigated extensively in bone-derived cells due to their sensitivity to the drug. While DHFR likely plays a role in normal cell response to MTX, research in this area is limited. Moreover, how MTX sensitivity differs among cell types responsible for maintaining connective tissues is unknown. The goal of this study was to investigate the role of DHFR and subsequent nucleotide synthesis in normal cell response to MTX. We also sought to compare adverse effects of MTX among normal cell types to identify sensitive populations and resistant cell sources for regenerative procedures targeting patients undergoing chemotherapy. DHFR overexpression or exogenous amino acid + nucleoside delivery rescued normal cells from adverse MTX effects. Conversely, DHFR knockdown impaired MTX-treated adipose-derived stem cell (ASC) osteogenesis. Proliferation of ASCs and bone marrow stem cells was more resistant to MTX than that of terminally differentiated osteoblasts. However, stem cells became susceptible to the drug after beginning differentiation. These results suggest that the ability of stem cells to survive and to maintain their surrounding tissues likely depends on whether they are in a “stem” state when exposed to MTX. Therapeutic strategies that delay the differentiation of stem cells until clearance of the drug may produce more favorable outcomes in the long-term health of treated tissues.

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Author Contributions: OSB, LEOD, and EMD designed the study and wrote the manuscript. OSB conducted all cell culture, western blots, PCR, and assays for proliferation and differentiation. LEOD conducted all plasmid synthesis work. VCF assisted with histological staining.

Keywords

mesenchymal stem cell; chemotherapy; regenerative medicine; methotrexate; cancer

Introduction

Chemotherapeutics are an effective treatment for many forms of cancer due to their targeting of rapidly dividing cells [1-4]. However, because of the non-selectivity of these drugs, normal (non-cancerous) cells also exhibit sensitivity to treatments [5]. Chemotherapy-induced impairment of normal connective tissue cells results in long-term damage, which can include tissue loss or dysfunction [6,7]. Methotrexate (MTX) is a commonly used chemotherapeutic agent known to harm both normal and cancerous cells through binding and competitive inhibition of dihydrofolate reductase (DHFR) [8]. DHFR is an essential protein responsible for reduction of folate and dihydrofolate to tetrahydrofolate, which is necessary for nucleotide production and subsequent DNA biosynthesis [9]. Since nucleotide production and DNA biosynthesis are critical for many cellular processes, treatment with MTX can lead to inhibited cell proliferation or apoptosis [10]. MTX has been employed to combat prevalent cancers, such as acute lymphoblastic leukemia and breast cancer [11,12]. The drug is also used long-term at low concentrations for treatment of rheumatoid arthritis and has recently been considered as a potential therapy to reduce obesity-related inflammation of adipose tissue [13,14]. While this drug is widely effective in the clinic, previous studies have found that MTX can cause impaired function of bone-derived cells, including osteoblasts (OBs) and bone marrow stem cells (BMSCs) [6,15,16]. High-dose treatment can lead to a reduction in OB and BMSC calcified matrix deposition and proliferation, which ultimately results in long-term bone loss and renders patients at risk for bone defects. Folinic acid or nucleosides can be delivered to patients undergoing MTX therapy to remedy the resulting nucleotide deficiencies and ameliorate toxicity [17,18]. However, few studies have been conducted in this field. Due to the link between cell impairment and long-term tissue damage, it is important to identify adverse effects of chemotherapeutics on cells that are responsible for tissue maintenance and function. Furthermore, since many normal cell types are also sensitive to chemotherapeutics, it is necessary to explore mechanistic or regenerative rescue approaches that can minimize adverse effects to normal cells and treat or prevent long-term tissue loss.

Adipose-derived stem cells (ASCs) are a regenerative cell population located in adipose tissue [19]. Due to their multilineage differentiation potential, these cells are being considered for use in a broad spectrum of cell-based therapies [20]. Within their niche, ASCs help regulate tissue metabolism and are responsible for tissue maintenance by replacing adipocytes during cell turnover and promoting angiogenesis [20-23]. Their clinical promise and significant regulatory role *in vivo* makes ASCs an important cell type to understand more completely. Unfortunately, not much is known about their response to harmful agents like MTX, which is an important consideration given the prevalence of MTX treatments prescribed in the clinic. Our group has previously shown that ASCs are relatively resistant to MTX when compared with a normal, non-stem cell fibroblast population [24]. We also determined that ASCs upregulate DHFR protein expression more than fibroblasts

during MTX treatment, potentially identifying a resistance mechanism that could be implemented in normal cells to prevent unwanted impairment. However, the role of DHFR in ASC MTX resistance is still not completely understood. Furthermore, little is known about how ASC MTX response compares with other normal cell types shown to be MTX-sensitive, like OBs and BMSCs [25]. Comparing the MTX response of ASCs with other cell types could reveal the extent of ASC MTX-resistance and potentially identify ASCs as a regenerative cell population capable of treating tissue loss after chemotherapy.

This study aimed to investigate how altering DHFR expression in non-stem and stem cell types influences their MTX response *in vitro*. We hypothesized that DHFR overexpression or exogenous amino acid + nucleoside delivery (GAT: glycine, adenosine, and thymidine) would increase resistance of MTX-sensitive cell types, like normal human fibroblasts (NHF) and osteoblasts (OBs). Additionally, we hypothesized that DHFR knockdown would induce drug susceptibility in normally MTX-resistant ASCs. To examine the role of DHFR and nucleotide synthesis in MTX-induced cell responses, NHFs were transfected with DHFR plasmids and then cell proliferation was monitored. As a more therapeutically relevant approach, GAT was delivered to normal cell types following MTX exposure to determine whether rescue occurred. To understand more about ASC MTX resistance, proliferation and differentiation potential were assessed after DHFR knockdown. Moreover, the MTX response of non-transfected ASCs was compared with that of bone marrow-derived stem cells (BMSCs) and OBs to evaluate differences in drug sensitivity among these stem and non-stem primary cell types.

Materials and Methods

Cell Types and Culture

Four different, primary cell types were used in this study: ASCs, NHFs, BMSCs, and OBs. All cells were isolated from human donors and used at low passage number. In most cases, a single donor was used, so interpretation was limited to phenomenological findings and the investigation of molecular mechanisms. Cells were maintained in humidified incubators at 37°C, 5% CO₂ and passaged at 80% confluence with 0.25% trypsin-EDTA (HyClone, GE Healthcare).

ASCs were isolated from human lipoaspirate following an established protocol [26] with minor modifications, as described previously [24]. Waste tissue was obtained from one, female donor (age 56) following procedures approved by the internal review board (IRB) at Rhode Island Hospital. ASCs were grown in expansion medium comprised of DMEM/F-12 (HyClone, GE Healthcare), 10% FBS (Zen-Bio), 1% antibiotic/antimycotic (HyClone, GE Healthcare), 0.25 ng/mL transforming growth factor- β 1, 5 ng/mL epidermal growth factor, and 1 ng/mL fibroblast growth factor (R&D Systems) [27]. Experiments used ASCs at passage 5.

NHFs isolated from neonatal human foreskins (a gift from Dr. Jeffrey Morgan) were expanded in high glucose DMEM (DMEM-HG, HyClone, GE Healthcare), 10% FBS (HyClone, GE Healthcare), and 1% penicillin/streptomycin (HyClone) [28]. Experiments used NHFs at passage 9.

BMSCs derived from one, female donor (age 20, a gift from Dr. Anita Shukla, purchased from Lonza, lot 0000305526) were cultured in α -MEM (HyClone, GE Healthcare), 15% FBS (HyClone, GE Healthcare), and 1% penicillin/streptomycin [29]. Experiments used BMSCs at passage 5.

OBs derived from human calvarial bone (a gift from Dr. Dioscaris Garcia, purchased from ScienCell) were grown in Osteoblast Medium (ScienCell) according to the manufacturer's instructions. Experiments used OBs at passage 5.

Plasmid Synthesis

Mammalian expression plasmids for transfection included pEmGFP-N1 (GFP control) and DHFR-mGFP (DHFR-GFP). pEmGFP-N1 was created via site-directed mutagenesis of pEGFP-N1 (Takara Clontech) to introduce the monomeric amino acid mutation A206K to EGFP [30]. Human DHFR cDNA (ORIGENE clone SC321334, Ref Seq: NM_000791.3) was inserted into the multiple cloning site of pEmGFP-N1 in frame with EmGFP via PCR-based cloning using Q5 high fidelity polymerase (New England BioLabs Inc.). The forward primer introduced a SalI site (5'-TAAGCAGTCGACGCCACCATGGTTGGTTCGCTAAAC-3') and the reverse primer introduced a (Gly-Ala)₄ linker and a BamHI site (5'-TGCTTAGGATCCGCCCTGCTCCTGCTCCAGCACCATCATTCTTCTCATATAC-3'). The fusion protein product has a 14 amino acid linker between the C-terminus of DHFR and the N-terminus of EGFP. The entire regions encompassing DHFR, linker, and EmGFP were sequence verified.

NHF Plasmid Transfection and Fluorescence-Activated Cell Sorting (FACS)

To assess the effects of DHFR overexpression on NHF MTX response, cells were transfected with DHFR-GFP or GFP control plasmids. Briefly, NHFs were uplifted, centrifuged, and resuspended at 10×10^6 cells/mL in 4°C DMEM-HG. Cells were transfected with either GFP or DHFR-GFP fusion expression plasmids (12.5 μ g DNA/mL) via electroporation using an Amaxa Nucleofector 2b device according to product literature (program T-016; Lonza). After recovering in complete medium for 10 min, cells were plated at $\sim 70,000$ cells/cm² in 6-well plates and incubated at 37°C overnight to allow for protein expression. Due to low transfection efficiencies (~ 10 -15%), fluorescence-activated cell sorting (FACS) was used to collect pure populations. Briefly, transfected cells were uplifted 24 h post-electroporation, resuspended at $\sim 0.5 \times 10^6$ cells/mL in Hank's Buffered Salt Solution (Hyclone, GE Healthcare), and filtered through a 40 μ m cell strainer. Cells were sorted using an Influx high-speed cell sorter (BD Biosciences) using a 488 nm laser and a 530/30 bandpass filter. Non-transfected cell populations were used as a control to gate forward and side scatter profiles as well as to define fluorescence intensity. Cells with fluorescent signal at least one order of magnitude over background noise was considered positive and were collected. Positively transfected NHFs were immediately plated at 8,000 cells/cm² in 96-well plates, and after adhering overnight, cultures were treated with 0-0.2 μ M MTX for 24 h. Cells were then lysed for protein extraction or were washed with 1 $\times$ PBS and expanded further for proliferation studies.

ASC siRNA Delivery

The role of DHFR in ASC MTX response was examined by silencing DHFR gene expression prior to MTX exposure. ASCs were plated in 96-well plates at 5,300 cells/cm² and allowed to adhere for 24 h. To achieve knockdown, cells were transfected for 24 h with 0.3 μ L/well RNAiMAX (Life Technologies) and either 50 nM non-targeting control (ON-TARGET plus Non-targeting Pool D-001810-10-05, Dharmacon) or *DHFR* (ON-TARGET plus Human *DHFR* siRNA-SMART pool L-008799-00-0010, Dharmacon) siRNA in antibiotic/antimycotic-free medium. 48 h post-transfection, a subset of cells was lysed to extract mRNA and protein, and the remaining cells were treated for 48 h with 0-50 μ M MTX. Cultures were washed with 1 $\times$ PBS following MTX removal, and fresh expansion or induction medium, was added, as described below.

Exogenous Amino Acid + Nucleoside (GAT) Treatment

NHFs—Temporal effects of GAT (glycine, adenosine, and thymidine) delivery on MTX-treated NHF proliferation were evaluated to optimize rescue regimen. NHFs were plated at 2,000 cells/cm² and allowed to adhere overnight. For temporal GAT studies, cells were treated with 0.2 μ M MTX for 24 h. During or after MTX treatment, cultures were exposed to one of four different GAT delivery regimens (Fig. 1): no GAT (medium was supplemented with DMEM-HG vehicle control), A GAT (GAT was added for the final 18 h of MTX treatment), B GAT (A GAT, plus cultures were supplemented again with GAT for 48 h immediately following MTX removal), and C GAT (GAT was added immediately following MTX removal for 48 h). GAT was used at final concentrations of 0.134 mM G (Fisher Scientific), 7.5 μ M A (Acros Organics), and 8.26 μ M T (Acros Organics) [31].

To assess the effects of GAT concentration on NHF MTX rescue, only the B GAT regimen was used. Cells were treated with physiologically relevant MTX concentrations ranging from 0.1 to 10 μ M MTX for 24 h [32]. Medium was supplemented with empirically determined GAT dosages. This included vehicle control, low-dose GAT (LD GAT; 0.067 mM G, 3.75 μ M A, 4.13 μ M T) or high-dose GAT (HD GAT; 0.67 mM G, 37.5 μ M A, 41.3 μ M T). MTX was removed, and wells were washed with 1 $\times$ PBS. Fresh expansion medium was then included with the final GAT dose.

ASCs, BMSCs, and OBs—GAT rescue effects on MTX-treated ASCs, BMSCs, and OBs were assessed using HD GAT, following the B GAT regimen. Cells were plated at 2,000 cells/cm² in 96-well plates. Cells were grown for 48-96 h to ensure entrance into log phase and then treated for 48 h with 10 μ M MTX, a concentration chosen for its clinical relevance in high dose chemotherapy regimens [32]. Media were supplemented with a vehicle control or HD GAT, similar to the B GAT regimen, as described previously. Upon MTX removal and 1 $\times$ PBS wash, the final 48 h GAT dose was added to wells along with either cell-specific expansion media for proliferation studies or induction media for differentiation studies, as described below.

Western Blot

Protein levels were assessed via western blot for DHFR overexpression and knockdown in NHFs and ASCs, respectively, following previously established protocols [24]. Equal

amounts of protein were separated on pre-cast SDS-PAGE gels (Bio-Rad) and transferred onto PVDF membranes (Millipore) before probing for DHFR (1:500, Abcam; ab133546) or β -Tubulin (1:1000, Developmental Studies Hybridoma Bank; E7-s). Blots were then developed using WesternSure PREMIUM Chemiluminescent Substrate (LI-COR) and scanned using a C-Digit Blot Scanner (LI-COR). Densitometry was used to semi-quantitatively assess protein levels using Image J (NIH). Experiments with ASCs and NHFs were done in 2 or 3 iterations, respectively, with a sample size of $n = 2-3$.

mRNA Extraction and qPCR

Effects of siRNA delivery on mRNA expression in ASCs were examined by qPCR. mRNA was extracted 48 h after cells were transfected with either non-targeting control or DHFR siRNA using a QuickRNA Mini Prep Kit (Zymo Research) following manufacturer instructions. Reverse transcription was then conducted using a SuperScript III First Strand cDNA Synthesis Kit (Life Technologies). Taqman Gene Expression Assay human primers for *DHFR* (Hs00758822_s1) and a reference gene, *GAPDH* (Hs03929097_g1) were used for amplification. Real-time fluorescence signals were then measured on a CFX Connect Real-Time PCR Detection System (BioRad Laboratories), and relative expression was determined using the comparative C_T method [33]. Experiments were conducted with 3 technical replicates.

Proliferation

Cell counts were used to reflect cell proliferation and indicate the relative health of the experimental groups. To quantify proliferation, cells were stained 48 h post-MTX removal with 5 μ g/mL Hoechst 33342 dye (Invitrogen, Life Technologies) for 15 minutes. Stained nuclei were imaged using a Cytation 3 reader (BioTek Instruments) and were quantified with Gen5 data analysis software (BioTek Instruments). Experiments were done in 3 iterations with a sample size of $n = 9-18$.

Multilineage Differentiation

To evaluate the effects of MTX and GAT rescue on differentiation, cells were induced along adipogenic (ASCs and BMSCs) or osteogenic (ASCs, BMSCs, and OBs) lineages after MTX treatment removal.

Adipogenic Differentiation—ASCs (siRNA transfected and non-transfected) and BMSCs were exposed to induction media [DMEM/F-12 (ASCs) or DMEM-HG (BMSCs), 10% FBS, 1 μ M dexamethasone, 10 μ M insulin, 0.5 μ M isobutyl-1-methylxanthine, 200 μ M indomethacin (Sigma), and 1% antibiotic/antimycotic] or control media [DMEM/F-12 (ASCs) or α -MEM (BMSCs), 10% FBS, 1% antibiotic/antimycotic] immediately following MTX removal along with the final 48 h GAT dose [34]. After two weeks of culture, samples were fixed with 3.7% paraformaldehyde (Thermo Fisher Scientific). Oil red O (ORO, Sigma) staining was used to visualize intracellular lipid droplets in adipogenic and control cultures. A custom MATLAB program was employed to quantify large lipid diameters and counts ($> 2 \mu$ m diameter), as previously described [35]. Experiments were done in 3 iterations with a sample size of $n = 9-18$.

Osteogenic Differentiation—ASCs and BMSCs were exposed to osteogenic induction media consisting of DMEM-HG (ASCs) or α -MEM (BMSCs), 10% FBS, 10 nM dexamethasone, 10 mM β -glycerophosphate, 0.15 mM ascorbate-2-phosphate, 10 nM 1,25-(OH)₂ vitamin D₃ (Sigma), and 1% antibiotic/antimycotic or control media consisting of DMEM/F-12 (ASCs) or α -MEM (BMSCs), 10% FBS, 1% antibiotic/antimycotic [36]. For OB “osteogenesis,” cells were exposed to induction medium consisting of α -MEM, 10% FBS, 0.1 μ M dexamethasone, 10 mM β -glycerophosphate, 0.15 mM ascorbate-2-phosphate, and 1% antibiotic/antimycotic or control medium, as described for BMSCs [37]. ASCs, BMSCs, and OBs were cultured before assessing either alkaline phosphatase (ALP) activity after 1 week or calcified matrix production after 3 weeks. After one week of induction, ALP activity was quantified using a Biovision ALP Activity Assay following manufacturer instructions [24]. After three weeks of culture, cells were fixed, and calcified matrix deposition was visualized using alizarin red S stain (ARS, Sigma). After imaging stained samples, ARS was eluted, and optical density was measured with spectrophotometry at 540 nm. Osteogenic data are presented on a per well basis. Experimental cell counts were determined by counting nuclei stained with 4',6-diamino-2-phenylindole (DAPI, Thermo Fisher Scientific, ARS) or Hoechst (ALP). Stained nuclei were quantified using the Cytation 3 reader. Experiments were done in 3 iterations with a sample size of $n = 8-14$.

Statistical Analysis

Two-way ANOVA was used to detect differences in proliferation among experimental groups for each individual cell type. For differentiation, two-way ANOVA was used to distinguish differences among experimental groups of induced conditions. Significance levels for individual comparisons were determined using a Tukey post-hoc test ($p < 0.05$ for significance). To control for non-experimental variability inherent in the differentiation assays, all data were normalized to the average value of individual runs for each experiment. Non-normal data sets were transformed prior to statistical analysis.

Results

NHF DHFR Overexpression

NHFs were transfected with GFP (control) or DHFR-GFP plasmids to investigate whether increasing DHFR expression would provide NHFs resistance to MTX. Transfection of NHFs with DHFR-GFP plasmids dramatically increased total DHFR protein expression when compared with endogenous DHFR levels from GFP-transfected cells, as revealed by western blot. Moreover, treatment with 0.1 μ M MTX for 24 h significantly increased both endogenous and plasmid DHFR expression by 110-120% (Fig. 2A, B, $p < 0.005$). Quantifying NHFs after treatment with 0.1 or 0.2 μ M MTX revealed that DHFR-GFP transfection led to a 30-40% increase in cell counts over control, GFP-transfected cells (Fig. 2C, $p < 0.001$). This overexpression resulted in no significant differences being observed between 0.1 μ M MTX treated and non-treated DHFR-GFP-expressing NHFs. However, treatment with 0.2 μ M MTX still impaired cell proliferation, leading to a 20% reduction compared to untreated samples ($p < 0.005$).

NHF GAT Rescue

To investigate the temporal effects of exogenous GAT delivery on NHF MTX rescue, cells treated with 0.2 μ M MTX for 24 h were exposed to several GAT regimens (Fig. 1). Comparing cell counts of untreated and MTX-treated groups revealed that MTX significantly reduced NHF counts by 30-70% in No GAT, A GAT, and C GAT conditions (Fig. 3A, $p < 0.001$). However, no significant differences were observed between untreated and MTX-treated counts in B GAT conditions.

To assess how GAT concentration influences NHF MTX rescue, MTX-treated cells (0.1-10 μ M) were exposed to either LD or HD GAT following the B GAT regimen (Fig. 3B). Within the No GAT condition, treatment with 0.1 μ M MTX significantly reduced cell counts from untreated controls by 60-90% ($p < 0.001$). For LD GAT treatment with 0.2-10 μ M MTX reduced NHF counts by 30-90% ($p < 0.001$). Conversely, for HD GAT conditions, no significant decreases were observed between untreated and MTX-treated cell counts at any concentration.

ASC DHFR Knockdown Effects

To examine how DHFR knockdown influences ASC MTX-resistance, ASCs were transfected with either control or DHFR siRNA, and their proliferative and differentiation potential was assessed following exposure to MTX. Western blot revealed that DHFR expression of siRNA-transfected cells, 48 h post-transfection, was reduced by 87%, consistent with an 85% knockdown of *DHFR* mRNA levels (Fig. 4A, B, C). ASC proliferation after MTX treatment (0.1-50 μ M) showed that neither MTX exposure nor DHFR knockdown without MTX treatment had an effect on cell numbers (Fig. 4D). ASC adipogenesis was similarly unaffected, with large lipid counts and diameters in adipogenic-induced samples being comparable between control and DHFR knockdown conditions, with no observable effects of MTX (Fig. 5 A, B). Conversely, ASC osteogenesis showed measurable changes in experimental groups. Treatment of ASCs with 0.1 μ M MTX significantly reduced ALP activity by 10-60% for both control and DHFR knockdown conditions (Fig. 5C, $p < 0.05$). Moreover, ALP activity in both untreated and MTX-treated samples was significantly reduced in DHFR knockdown conditions from 3-20% ($p < 0.01$).

MTX Effects on ASC, BMSC, and OB Proliferation

To evaluate differences in MTX response and to assess the rescue potential of GAT delivery, proliferation of MTX-treated ASCs, BMSCs, and OBs was examined. Without GAT delivery, MTX treatment had no effect on ASCs or BMSCs but significantly reduced OB cell counts by 30% (Fig. 6, $p < 0.001$). GAT delivery alone also reduced OB cell counts to a similar degree ($p < 0.001$). Interestingly, MTX treatment under these conditions did not result in a further decrease in OB cell counts. GAT as a factor also slightly reduced ASC counts from No GAT conditions by 6%-15% in untreated and MTX-treated groups ($p < 0.05$). Conversely, no significant differences were observed for BMSCs.

MTX Effects on ASC, BMSC, and OB Differentiation

To examine how MTX treatment and GAT delivery influenced ASC, BMSC, and OB differentiation potential, MTX-treated cells were induced along adipogenic (ASCs, BMSCs)

or osteogenic (ASCs, BMSCs, OBs) lineages. Imaging of adipogenic cultures showed similar ORO staining among induced conditions within ASC and BMSC sample groups (Fig. 7A, B). Quantification of ORO revealed that large lipid counts in ASCs were comparable between untreated and MTX-treated conditions (Fig. 7C). However, BMSC large lipid counts were increased by 13-20% after MTX treatment ($p < 0.05$). Measurement of large lipid diameter showed that both ASC and BMSC values were similar between their respective untreated and MTX-treated conditions (Fig. 7D). GAT had no effect on MSC adipogenesis.

Examination of osteogenic cultures revealed that without GAT, MTX treatment reduced ASC and OB ALP activity by 30% (Fig. 8A, $p < 0.01$). No significant differences were observed in BMSC samples. Evaluation of ARS staining showed that MTX treatment reduced calcified matrix production of OBs by 50% in No GAT conditions (Fig. 8B, $p < 0.005$). A decrease in calcified matrix, although not significant, was also observed for ASCs. Similarly, no significant MTX effects were observed for BMSC calcified matrix production. In the absence of MTX, GAT delivery adversely affected ALP activity in ASCs. However, for all cell types undergoing osteogenesis in the presence of MTX, exogenous GAT delivery resulted in ALP and ARS levels comparable to the untreated/No GAT conditions, indicating a rescue from adverse MTX effects.

Discussion

The results of this study indicate that DHFR, and subsequent nucleotide synthesis, is central to the MTX response of non-stem cells but less critical for mesenchymal stem cells (MSCs). DHFR overexpression or exogenous GAT delivery rescued sensitive, normal cells from adverse MTX effects. Furthermore, differences were observed for mature versus stem cell response to MTX, with undifferentiated ASCs and BMSCs exhibiting greater resistance than terminally differentiated OBs. These data suggest that sensitivity to MTX, as assessed by proliferation and differentiation, is influenced by DHFR expression and dependent on cell type.

Proliferation experiments of MTX-treated NHFs revealed that DHFR-transfected cell counts were higher than cells transfected with GFP. These results indicate that DHFR overexpression reduces adverse effects of MTX but suggest that NHF proliferation is still MTX-susceptible if insufficient DHFR levels are present in the cell. Arnoldo and colleagues observed similar results when they overexpressed DHFR in HEK cells, indicating that low DHFR expression renders multiple cell types sensitive to the drug [38]. Furthermore, it has been reported that DHFR overexpression is a MTX-resistance mechanism of leukemia cells, confirming that non-stem cell response to MTX is often DHFR-related [39]. While DHFR-transfected NHFs were resistant to 0.1 μM MTX, treatment with 0.2 μM MTX significantly reduced cell counts from untreated controls. These results indicate that while overexpression of DHFR is capable of rescuing NHFs at low MTX concentrations, there is insufficient plasmid-generated DHFR to rescue cells at higher MTX concentrations. As such, rescue with DHFR overexpression is limited and would be difficult to implement for clinical applications. While a more efficient promoter in the plasmid might address this issue, lack of

safe and efficacious *in vivo* transfection approaches also restrict the practical use of this approach to protect normal cells [40].

Due to the limitations observed with MTX rescue based on DHFR expression levels, exogenous GAT delivery was investigated as a more clinically relevant option. Previous reports have indicated that exposure to exogenous amino acids, purines, and pyrimidines can remedy the deficiencies that result from MTX treatment and can thus rescue cells from adverse effects [31,41]. Temporal GAT studies with NHFs found that exposure to GAT for the final 18 h of MTX treatment (A GAT), or exposure for the 48 h following MTX removal (C GAT) were inadequate rescue regimens. However, when combined (B GAT), GAT successfully prevented MTX impairment of NHF proliferation. This illustrates that adverse MTX effects are irreversible by 24 h, as has been reported previously [42], but also that damage continues to occur after MTX is removed. Continued cellular damage after MTX removal is hypothesized to be due to formation of MTX polyglutamates [43]. These long MTX polyglutamate chains can be retained by cells for days following treatment and continue to cause damage [44].

Not surprisingly, higher GAT amounts were found to be necessary to rescue NHFs exposed to higher MTX concentrations. These results are consistent with previous literature and indicate that low amounts of GAT do not adequately remedy the large purine and pyrimidine deficiencies that occur at higher MTX concentrations [45]. As such, we have shown that GAT rescue must be tuned to the level of MTX administered. However, while high doses of GAT did not adversely affect non-MTX-treated NHFs, proliferation and differentiation studies revealed that excess GAT was detrimental to ASC and OB properties. In these cases, GAT damage is likely due to a surplus of thymidine, which can lead to accumulation of thymidine triphosphate and restrict DNA synthesis [46-48]. Therefore, even in the presence of MTX, excess GAT can impair non-stem and stem cells, with susceptibility varying by cell type. In the case of OB proliferation, there was no additive (negative) effect of GAT and MTX. Since MTX prevents nucleotide synthesis, its presence could bring the thymidylatedown to non-toxic levels, offsetting GAT treatment. To minimize unintended damage, GAT may be most suitable for protecting cells exposed only to low MTX concentrations, similar to levels given for rheumatoid arthritis rather than acute chemotherapy, since less harmful levels of GAT could be delivered and still prevent nucleotide deficiency. Furthermore, to ensure that GAT is a viable rescue technique for these treatment types, additional studies should be conducted to confirm that GAT does not also rescue cancerous cells and is more effective than leucovorin, the current rescue approach [49].

Unlike NHFs, undifferentiated ASCs exhibited resistance to MTX; however, when placed in an osteogenic environment, ALP activity decreased, suggesting the onset of susceptibility. Osteogenic induction assays revealed that treatment with MTX decreased ASC ALP activity, with or without DHFR knockdown, at concentrations as low as 0.1 μ M. In differentiating ASCs, ALP activity was also slightly reduced by DHFR knockdown in both untreated and MTX-treated conditions, suggesting that expression levels of this protein can influence osteogenesis. Multiple reports have shown that osteogenic capacity of bone-derived cells is impaired by MTX treatment and leads to long-term effects, such as osteoporosis and bone

defects [16,50]. However, to our knowledge, no studies have looked at how DHFR knockdown alone influences osteogenic capacity and/or differentiation, which is relevant since MTX binds and inhibits this protein. Our data showing the importance of high DHFR expression for osteogenesis indicate a potential reason as to why this tissue lineage is especially susceptible to MTX effects. Moreover, our results further support investigating whether upregulation of DHFR in normal osteoblasts can help prevent bone loss in patients undergoing chemotherapy.

However, only a subset of ASC characteristics was observed to be dependent on DHFR and sensitive to MTX. While ASC osteogenesis was influenced by DHFR protein levels, ASC adipogenesis and proliferation were not. Thus, DHFR expression is not the sole regulator of MTX resistance for ASC proliferation or adipogenesis. The data also suggest that ASCs do not depend on high levels of DHFR to divide or undergo adipogenic differentiation, which may indicate an additional mechanism of MTX resistance. To our knowledge, this is the first study to investigate the role of DHFR in ASC function. These results are important for understanding how necessary bioprocesses are regulated within ASCs. Moreover, they provide encouraging insight for adipogenic reconstructive procedures using autologous cells targeting patients undergoing MTX therapy.

Since ASCs exhibited resistance to MTX until undergoing osteogenesis, stemness could serve as an alternative mechanism of resistance. BMSC MTX response further supports this hypothesis, being that no adverse drug effects were observed until adipogenic induction. A previous report investigating cancer stem cells also considered this theory, since it was observed that acquisition of stemness can lead to drug resistance [51]. Our data suggest that resistance to chemotherapeutics might not just be limited to cancer “stem” cells but might extend to healthy stem cell types as well. Investigation into the expression of stemness factors common between these abnormal and normal cells might reveal targets capable of reducing cancer stem cell drug resistance. These results are also important for developing regenerative therapies to treat bone loss in patients undergoing chemotherapy. While MSCs may be potential cell sources for treatment of drug-induced tissue damage, their regenerative properties could be limited if MTX is still present within the microenvironment when they start differentiating.

Comparing the MTX response of ASCs, BMSCs, and terminally differentiated OBs further supported a potential role for stemness in MTX resistance. Proliferation assays showed that MTX treatment significantly reduced the cell counts of terminally differentiated OBs, while ASCs and BMSCs were unaffected, which is consistent with previous reports [25,32,52]. These data indicate that OBs, like NHFs, are more susceptible to adverse MTX effects than stem cells with regards to proliferation, potentially due to their differentiated state. In addition to stemness, an explanation for these differences could lie partly in their variable proliferation rates, with OBs exhibiting faster population doubling times than ASCs and BMSCs (Table 1). Since chemotherapeutic agents like MTX target rapidly dividing cells, OBs may be inherently more susceptible to the drug than cells that divide more slowly [4]. Alternatively, OB MTX sensitivity could be a result of their primary reliance on DHFR for cell division. Since knockdown experiments in the current study showed that DHFR

expression influenced ASC osteogenesis, it is possible that proliferation of mature osteoblasts might also be regulated in part by DHFR.

While proliferation assays indicated stem cells possessed a superior resistance to MTX, differentiation assays revealed that this resistance was not absolute. MTX treatment impaired osteogenesis to varying degrees in ASCs and OBs, while BMSCs exhibited no change, which is consistent with previous studies [16,50,53]. Interestingly, adverse MTX effects could be ameliorated by GAT delivery. These data indicate that DHFR function, and subsequent nucleotide synthesis, likely contributes to ASC and OB osteogenesis but does not influence BMSCs. Understanding differences in drug sensitivity and how MSCs regulate differentiation is important for optimizing regenerative therapies to treat drug-induced damage. Furthermore, our data suggest that impaired OB function may contribute more to MTX-induced bone loss than any effect on BMSCs. It should be noted that on a per cell basis, MTX had no adverse effects on ASC osteogenesis (Supplemental Fig. 1). These results are due to a reduction in cell numbers as differentiating ASCs become MTX-susceptible following induction and suggest that the osteogenic capacity of surviving stem cells is retained. Cells that do not survive are hypothesized to be differentiating rapidly, increasing their susceptibility to any remaining MTX polyglutamates. It is possible that more cells would resist adverse MTX effects if differentiation was delayed after treatment. However, this is not always possible in clinical applications of MTX.

When examining the effects of MTX on adipogenic differentiation potential, ASC adipogenesis did not change, and BMSC adipogenesis experienced a minor increase in the number of large, intracellular lipids when MTX was present. These data hint that there may be differences in ASC and BMSC MTX sensitivity and that DHFR may play a larger role in adipogenesis of BMSCs than ASCs. Georgio and colleagues have also reported that MTX increases the adipogenic potential of BMSCs in a rat model [16]. They later found that this response is due to a reduction in β -catenin expression, a regulator of adipogenic and osteogenic differentiation [54]. Reports on ASCs have also shown that knockdown of β -catenin expression increases adipogenic potential. However, it is possible that while MTX reduces β -catenin expression in BMSCs, it does not affect expression in ASCs. Further investigation into the interaction between DHFR and β -catenin in these two cell types is necessary to confirm this hypothesis. Since low β -catenin expression has also been shown to reduce osteogenic potential in MSCs, it would be interesting to see if this protein, in conjunction with DHFR, contributes to the MTX-induced osteogenic reduction observed for ASCs [54]. Since ASC adipogenesis is not adversely affected by MTX when compared with BMSCs, adipose tissue might not be as susceptible as bone to MTX-induced tissue loss.

Although the 24-48 h MTX treatment regimens used in this study effectively revealed adverse consequences of the drug, longer treatments should also be considered to better mimic repeated chemotherapy doses. Our findings revealed that BMSC proliferation was resistant to 48 h of 10 μ M MTX treatment. However, previous reports have cited that 0.001-1 μ M treatments lasting 15-66 days, mimicking long-term rheumatoid arthritis therapy, do impair BMSC division [15,55]. Therefore, it is important to consider that use of longer MTX treatments could have yielded different results. An additional, influential factor is how the *in vivo* environment affects cellular drug sensitivity. While *in vitro* studies are

imperative for understanding the mechanistic effects of MTX on cell function, it has been reported that discrepancies can exist between *in vitro* and *in vivo* drug effects. For example, Georgiou and colleagues found that *in vivo*, BMSC osteogenic potential is reduced by MTX but remains unchanged *in vitro*, similar to our results here [16]. The authors concluded that the microenvironment plays a role in stem cell susceptibility to MTX. As such, stem cells might have been more susceptible to adverse MTX effects, had they been treated within their niches.

The current study demonstrated that both DHFR expression and stemness can influence MTX response. Findings revealed that increased DHFR expression or exogenous nucleoside delivery could rescue non-stem cell types from adverse MTX effects, while DHFR knockdown sensitized ASCs to the drug only during osteogenesis. Understanding mechanisms behind normal cell MTX response is crucial for identifying targets for potential rescue techniques. Results also showed that non-stem cells are more susceptible to adverse MTX effects than MSCs, providing an explanation for MTX-induced bone tissue damage in cancer patients. Importantly, this also raises further concerns for patients undergoing MTX-based chemotherapy. The resistant characteristics of MSCs provides some hope, particularly if they can be used to prevent or regenerate healthy tissue loss during chemotherapeutic treatment.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1.

Exogenous amino acid + nucleoside (GAT) delivery regimen schematic. (A) GAT was delivered to MTX-treated NHF samples following four different regimens. (B) Either No GAT or B GAT schedules were used during the 48 h MTX treatment for ASCs, BMSCs, and OBs.

Figure 2.

Effects of MTX on GFP and DHFR-GFP-transfected NHFs. (A) Protein expression for GFP and DHFR-GFP-transfected NHFs after treatment with 0.1 μM MTX. (B) Quantification of band intensity revealed that DHFR expression was significantly increased based on MTX as a factor ($p < 0.005$), but was not significant for individual comparisons. Quantified protein expression is presented as a percent of untreated, endogenous DHFR expression in GFP-transfected cells. (C) Cell counts of DHFR-GFP-transfected NHFs were significantly higher than GFP-transfected cells after MTX treatment ($p < 0.001$). While 0.1 μM MTX-treated, DHFR-GFP-transfected NHF counts were similar to untreated controls, 0.2 μM MTX-treated counts were significantly reduced ($p < 0.005$). Cell counts are presented as a fold difference of untreated, GFP-transfected control counts. All error bars depict standard deviation. Groups with different letters are significantly different ($p < 0.05$).

Figure 3.

Temporal and concentration effects of nucleoside/GAT delivery for rescue of MTX-treated NHFs. (A) GAT delivery regimens rescued cell numbers to varying degrees, with B GAT providing complete recovery. (B) Successful GAT rescue depended on both the concentration of MTX and concentration of GAT. Too little GAT resulted in incomplete rescue of cell numbers, although some recovery could be observed for lower MTX concentrations. Cell counts are presented as a fold difference of untreated, No GAT counts. Error bars depict standard deviation. Groups with different letters are significantly different ($p < 0.05$).

Figure 4.

Effect of DHFR knockdown on MTX-treated ASC proliferation. (A) Protein expression from control and DHFR siRNA-treated ASCs. (B) Quantification of band intensity showed an 87% reduction in DHFR protein levels after knockdown. (C) qPCR confirmed the effectiveness of DHFR siRNA transfection, also showing a 85% reduction in mRNA expression. (D) DHFR knockdown had no effect on ASC proliferation, regardless of MTX concentration. Protein and mRNA expression are presented as a fold difference of control siRNA samples. Cell counts are presented as a percent of untreated counts in control siRNA samples. Error bars depict standard deviation.

Figure 5.

Effects of DHFR knockdown on MTX-treated, ASC differentiation. Quantification of large (A) lipid count and (B) diameter revealed no differences between siRNA types or among MTX concentrations for adipogenic differentiation. (C) For osteogenic differentiation, treatment with 0.1 μM MTX and knockdown of DHFR both reduced ALP activity of ASCs ($p < 0.05$ and 0.01 , respectively). No individual comparisons showed statistically significant differences. Values are presented as a fold difference of untreated levels within non-targeting control siRNA conditions. Error bars depict standard deviation. Groups with different letters are significantly different ($p < 0.05$).

Figure 6.

Proliferation of MTX-treated ASCs, BMSCs, and OBs. For ASCs, MTX had no effect on proliferation, but overall cell counts were significantly reduced based on GAT as a factor ($p < 0.05$). No effect was observed for individual comparisons. For BMSCs, neither MTX nor GAT affected cell counts. For OBs, proliferation was adversely affected by both MTX treatment and GAT delivery ($p < 0.001$), but these effects were not additive. Cell counts are presented as a fold difference of untreated/No GAT control counts. Error bars depict standard deviation. Groups with different letters are significantly different ($p < 0.05$).

Figure 7.

Adipogenic potential of MTX-treated ASCs and BMSCs. (A, B) Imaging of oil red O staining revealed similar amounts of lipid production among groups for ASCs and BMSCs. (C) Quantification of large lipid counts showed both MTX and GAT had no effect on ASC adipogenesis. Conversely, MTX treatment significantly increased large lipid counts of BMSC samples for both No GAT and GAT conditions ($p < 0.05$) but was not significant for individual comparisons. (D) MTX treatment did not affect large lipid diameter in ASC or BMSC cultures. Large lipid count and diameter are presented as a fold difference of ASC or BMSC untreated/No GAT control values, respectively. Error bars depict standard deviation. (Scale bar indicates 200 μ m).

Figure 8.

Osteogenic capacity of MTX-treated ASCs, BMSCs, and OBs. (A) MTX treatment significantly reduced the ALP activity of ASCs and OBs ($p < 0.01$) in No GAT conditions but did not affect BMSCs. No significant differences were observed between untreated/No GAT and MTX-treated/GAT conditions for any cell type, indicating complete rescue. (B) MTX significantly reduced the calcified matrix production of OBs ($p < 0.005$) but had no significant effect on ASCs or BMSCs. As for ALP activity, no differences were observed between untreated/No GAT and MTX-treated/GAT conditions for any cell type. Values are presented as a fold difference of ASC, BMSC, or OB untreated/No GAT control levels, respectively. Error bars represent standard deviation. Groups with different letters are significantly different ($p < 0.05$).

Table 1
Population Doubling Times of Normal Cells

Cell Type	Population Doubling Time (h)
ASC	21
BMSC	51
NHF	24
OB	13