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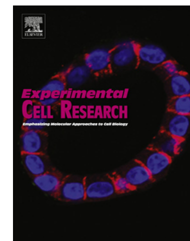

Research Article

Adipose-derived stem cells retain their regenerative potential after methotrexate treatment

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ABSTRACT

In musculoskeletal tissues like bone, chemotherapy can impair progenitor cell differentiation and proliferation, resulting in decreased bone growth and mineralization throughout a patient's lifetime. In the current study, we investigated the effects of chemotherapeutics on adipose-derived stem cell (ASC) function to determine whether this cell source could be a candidate for repairing, or even preventing, chemotherapy-induced tissue damage. Dose-dependent proliferation rates of ASCs and normal human fibroblasts (NHFs) were quantified after treatment with cytarabine (CY), etoposide (ETO), methotrexate (MTX), and vincristine (VIN) using a fluorescence-based assay. The influence of MTX on the multipotency of ASCs and freshly isolated stromal vascular fraction (SVF) cells was also evaluated using lineage-specific stains and spectrophotometry. ASC and NHF proliferation were equally inhibited by exposure to CY and ETO; however, when treated with MTX and VIN, ASCs exhibited greater resistance. This was especially apparent for MTX-treated samples, with ASC proliferation showing no inhibition for clinically relevant MTX doses ranging from 0.1 to 50 μM. Additional experiments revealed that the differentiation potential of ASCs was not affected by MTX treatment and that upregulation of dihydrofolate reductase possibly contributed to this response. Moreover, SVF cells, which include ASCs, exhibited similar resistance to MTX impairment, with respect to cellular proliferation, clonogenicity, and differentiation capability. Therefore, we have shown that the regenerative properties of ASCs resist the cytotoxicity of MTX, identifying these cells as a potential key for repairing musculoskeletal damage in patients undergoing chemotherapy.

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Abbreviations: ASC, adipose-derived stem cell; BMSC, bone marrow-derived stem cell; MTX, methotrexate; CY, cytarabine; ETO, etoposide; VIN, vincristine; SVF, stromal vascular fraction; PBS, phosphate buffered saline; ORO, Oil Red O; ARS, Alizarin Red S; sGAG, sulfated glycosaminoglycan; DMMB, dimethylmethylene blue; CFU, colony forming unit; DHFR, dihydrofolate reductase

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Introduction

The influence of chemotherapy on cellular function is of increasing interest to the stem cell field. While the effects of chemotherapeutics on bone marrow stem cells (BMSCs) and hematopoietic stem cells have been studied for a number of years [12,21], researchers have only recently begun investigating the effects of these drugs on adipose-derived stem cells (ASCs) [23,42]. Advancements in drug formulations have resulted in increased patient survival, but unfortunately many of these chemotherapeutics also have severe side effects, such as long-term musculoskeletal damage [31]. Reports suggest that these impairments result from the slowed proliferation, restricted differentiation, and apoptosis of progenitor cells, such as BMSCs [14]. Investigating the influence of chemotherapeutics on other stem cell types, like ASCs, will help identify whether these adverse side effects are universal or whether susceptibility is specific to BMSCs. Furthermore, the possibility of identifying alternative mesenchymal stem cell sources resistant to the cytotoxic effects of these drugs would provide a means to treat, or even prevent, tissue damage. The regenerative properties of ASCs make them an attractive cell population for these therapies. Moreover, they have been shown to withstand other toxic environments like oxidative stress, suggesting that ASCs might also resist functional damage induced by chemotherapeutics [7].

Only limited investigations have been made into the area of mesenchymal stem cell response to chemotherapeutics [23,27]. The effects of these treatments on ASC proliferation and differentiation potential have not previously been investigated thoroughly. Differences in mechanism of action suggest that, like BMSCs, ASCs will vary in their susceptibility to chemotherapeutic agents [21]. Therefore, it is necessary to evaluate many commonly used drugs in order to understand the impact of each individual treatment on ASC regenerative properties. Comparing chemotherapeutic effects on ASCs versus a normal, non-stem, somatic cell type provides a simple way to identify resistance or susceptibility to a given drug since neither cell type is directly targeted.

The goal of this study was to evaluate the effects of methotrexate (MTX), vincristine (VIN), cytarabine (CY), and etoposide (ETO) on ASC regenerative properties. Chemotherapeutic agents were chosen based on their diverse mechanisms of action and their application towards a wide range of cancers. MTX functions by binding to and inhibiting dihydrofolate reductase (DHFR), an essential protein for DNA synthesis [6]. Conversely, VIN prevents microtubule formation, CY inhibits polymerase function, and ETO targets topoisomerase II [13,28,29]. After treatment with individual chemotherapeutics *in vitro*, ASC growth was compared with untreated ASCs and treated/untreated normal human fibroblasts (NHFs). These comparisons enabled us to identify significant deviation from normal growth trends and evaluate the resistance of ASCs to chemotherapeutics relative to untargeted, non-stem somatic cells. We also assessed how multilineage differentiation potential was affected by drugs that did not inhibit ASC proliferation, and investigated the possible mechanism behind this immunity. These results provide important insight towards understanding how ASC regenerative properties are affected by commonly used chemotherapeutics and identify mechanisms of chemotherapeutic resistance that could be useful in designing regenerative therapies for cancer patients.

Materials and methods

Cell culture

ASCs, originally isolated from the subcutaneous fat of healthy human, non-diabetic, non-smoking female donors, aged 18–60 years ($N=7$), were purchased from Zen-Bio, Inc. (superlot #36; Research Triangle Park, NC). ASCs were grown in expansion medium consisting of DMEM/F-12 (HyClone), 10% FBS (Zen-Bio), 1% antibiotic/antimycotic (HyClone), 0.25 ng/mL transforming growth factor-β1, 5 ng/mL epidermal growth factor, and 1 ng/mL fibroblast growth factor (R&D Systems) [9]. ASCs were maintained in humidified incubators at 37 °C, 5% CO_2 and passaged at 80% confluence with 0.25% trypsin-EDTA (HyClone). Experiments used ASCs at passage 4–6 (P4–6).

NHFs derived from neonatal human foreskins (a gift from Dr. Jeffrey Morgan) were expanded in high glucose DMEM (DMEM-HG, HyClone), 10% FBS (HyClone), and 1% penicillin/streptomycin (HyClone) [39]. Experiments used NHFs at P6–9.

Isolation and culture of stromal vascular fraction (SVF) cells

All procedures were approved by the institutional review board (IRB) at Rhode Island Hospital. Human lipoaspirate was obtained from female donors, aged 20–56 ($N=3$). SVF cells were isolated using established protocols [8]. Briefly, liposuction waste tissue was washed thoroughly with phosphate-buffered saline (PBS) and digested for one hour at 37 °C in an equal volume of 0.1% type I collagenase (Worthington Biochemical Corporation). Samples were centrifuged at 300 g for 5 min, and the pellet was resuspended in stromal medium (DMEM/F-12, 10% FBS, 1% antibiotic/antimycotic) to neutralize the enzyme. Cells were washed twice more and incubated for 10 min at room temperature in an erythrocyte lysis buffer (155 mM NH_4Cl, 10 mM K_2CO_3, and 0.1 mM EDTA) to remove red blood cells. The remaining cell population was centrifuged and resuspended in stromal medium, then filtered sequentially through 100 μM and 70 μM strainers before cell counting. All samples were stored in liquid nitrogen using freezing medium containing 80% FBS, 10% dimethyl sulfoxide, and 10% expansion medium. Prior to experimentation, cells were quickly thawed and plated in expansion medium at 2000 cells/cm^2. SVF cells were treated with chemotherapeutics at P0. Clonogenicity and differentiation were assessed at P1.

Chemotherapeutic agents

The following chemotherapeutics (and concentrations) were used in our experiments: MTX (0.1–50 μM, MP Biomedicals), VIN (0.01–1 μM, Cayman Chemicals, Ann Arbor, Michigan), CY (1–100 μM, Accela ChemBio, Shanghai, China), and ETO (0.1–5 μM, MP Biomedicals). The concentration range for each drug was chosen based on clinically relevant doses reported in the plasma of patients receiving chemotherapy [21]. While treatment times ranging from 48–72 h have been used in the past [14,21,27], a duration of 24 h was used in the current study based on pilot work showing a dramatic response of ASCs and NHFs to the listed chemotherapeutics within that time frame.

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