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Melphalan exposure induces an interleukin-6 deficit in bone marrow stromal cells and osteoblasts

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ABSTRACT

Bone marrow stromal cells (BMSC) and osteoblasts are critical components of the microenvironment that support hematopoietic recovery following bone marrow transplantation. Aggressive chemotherapy not only affects tumor cells, but also influences additional structural and functional components of the microenvironment. Successful reconstitution of hematopoiesis following stem cell or bone marrow transplantation after aggressive chemotherapy is dependent upon components of the microenvironment maintaining their supportive function. This includes secretion of soluble factors and expression of cellular adhesion molecules that impact on development of hematopoietic cells. In the current study, we investigated the effects of chemotherapy treatment on BMSC and human osteoblast (HOB) expression of interleukin-6 (IL-6) as one regulatory factor.

IL-6 is a pleiotropic cytokine which has diverse effects on hematopoietic cell development. In the current study we demonstrate that exposure of BMSC or HOB to melphalan leads to decreases in IL-6 protein expression. Decreased IL-6 protein is the most pronounced following melphalan exposure compared to several other chemotherapeutic agents tested. We also observed that melphalan decreased IL-6 mRNA in both BMSC and HOB. Finally, using a model of BMSC or HOB co-cultured with myeloma cells exposed to melphalan, we observed that IL-6 protein was also decreased, consistent with treatment of adherent cells alone. Collectively, these observations are of dual significance. First, suggesting that chemotherapy induced IL-6 deficits in the bone marrow occur which may result in defective hematopoietic support of early progenitor cells. In contrast, the decrease in IL-6 protein may be a beneficial mechanism by which melphalan acts as a valuable therapeutic agent for treatment of multiple myeloma, where IL-6 present in the bone marrow acts as a proliferative factor and contributes to disease progression. Taken together, these data emphasize the responsiveness of the microenvironment to diverse stress that is important to consider in therapeutic settings.

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1. Introduction

The ability of the supportive cells of the bone marrow microenvironment, including bone marrow stromal cells (BMSC) and osteoblasts that comprise the endosteal niche, to maintain their functional integrity following chemotherapy or irradiation is vital for efficient reconstitution of hematopoiesis. The importance of specialized niches within the marrow environment that support stem cell self-renewal and a supply of mature blood cells has been described in detail [1–4].

Several groups have documented chemotherapy-induced stromal cell damage [5–7]. In addition, BMSCs isolated from patients receiving standard chemotherapy regimens had a reduced capacity to form confluent monolayers [5]. Chemotherapy-induced damage diminishes the ability of the BMSCs to self-repair, ultimately leading to decreased numbers of functional mature blood cells [6]. Galotto et al. demonstrated that the patients receiving allogeneic bone marrow transplants have irreversible stromal damage measured using functional assays that showed CFU-F frequencies did not recover to normal levels even after 12 years post-transplant

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[7]. These investigations emphasize the vulnerability of the components of the endosteal niche.

Interleukin-6 (IL-6) is a pleiotropic cytokine that has important roles in expansion of hematopoietic progenitors, induction of acute-phase proteins for immune and inflammatory responses, and regulation of bone metabolism [8,9]. IL-6 is secreted from BMSCs and osteoblasts, and has both proliferative and anti-proliferative effects. In the bone marrow microenvironment, IL-6 regulates B-cell differentiation and stimulation of T-cells, both necessary to maintain the immune system [10]. An IL-6 deficiency in the microenvironment decreases DNA synthesis in normal hematopoietic progenitor cells [11]. Long-term bone marrow cultures established from IL-6 knockout mice had delayed stromal cell layer development [11]. Additionally, reduced hematopoietic support activity, measured by CFU-GM, BFU-E, and cobblestone areas, which are characteristic of active hematopoietic proliferation was noted in the absence of IL-6 as well [11]. Moreover, IL-6 deficient mice have impaired immune and acute-phase responses [12]. IL-6 deficient mice challenged with diverse viruses and pathogens demonstrated acute-phase inflammatory responses were compromised [12]. Relevant to the current study, Patchen et al. observed that IL-6 administration following radiotherapy accelerated hematopoietic recovery in a murine model [13]. Based on the association of IL-6 deficits with sub-optimal hematopoietic recovery, we undertook investigation to determine whether chemotherapy dysregulates IL-6 expression in BMSC and osteoblasts as one factor which might be involved in the dysregulated hematopoietic support capacity of the bone marrow microenvironment following dose-escalated chemotherapy that has been described by others [5–7].

In the current model of chemotherapy-induced damage we included the chemotherapeutic agent melphalan. Melphalan is extensively used in pre-transplant chemotherapy regimens for autologous and allogeneic stem cell transplantations [14,15]. As such, damage imposed by it on the microenvironment is of pronounced clinical relevance.

Our results indicated that BMSCs and osteoblasts express diminished IL-6 protein following chemotherapy exposure with melphalan. DNA damage accumulation by interruption of repair did not result in the same magnitude of decrease in IL-6 as that observed following melphalan exposure, suggesting that there is some specificity to the melphalan induced change that exceeds general DNA damage effects. In addition, in a co-culture model of BMSC and HOB with myeloma cells, melphalan exposure sustained its ability to decrease IL-6 protein, suggesting melphalan's effect is sustained in a microenvironment that is typical of tumor and stromal or osteoblast interactions. Collectively, our data suggest that melphalan treatment induces an IL-6 deficit in BMSCs and osteoblasts of the endosteal niche, which may contribute to diminished ability of the bone marrow microenvironment to support reconstitution of hematopoiesis following transplantation. Additionally, melphalan's effect is maintained even in the presence of myeloma tumor cells, potentially contributing to its efficacy in this disease setting. As such, both the negative and positive effects of this alkylating agent via its influence on the marrow microenvironment are highlighted in the distinct settings of hematopoietic support as well as a tumor sanctuary site.

2. Materials and methods

2.1. Cell culture and reagents

Primary human BMSC were derived from consenting donors with the approval of the West Virginia University Institutional Review Board. These cells are cultured to morphological homogeneity and are characterized by their constitutive expression of VCAM-1 as well as GM-CSF, kit-ligand and other hematopoietic factors. In addition, the BMSC support IL-7 dependent pro-B cells. BMSC were maintained in alpha-modification of Eagle's medium (α-MEM) supplemented with 2 mM L-glutamine, 10% fetal bovine serum (Hyclone, Logan, UT), 100 μg/ml streptomycin, 100 IU/ml penicillin and 5×10^{-5} M 2-β mercaptoethanol at 37 °C in 6% CO_2. Primary human osteoblasts (HOB) were obtained from PromoCell (Heidelberg, Germany) and maintained in osteoblast growth media with osteoblast supplement as recommended by the manufacturer. HOB are isolated from normal femoral bone tissue from the hip and knee region. Phenotype of the osteoblasts was confirmed by alkaline phosphatase staining and bone mineralization assays. The HOB also express osteocalcin and collagen 1A1 consistent with the typical osteoblast phenotype. In experiments that include chemotherapy exposure, melphalan (Sigma Aldrich, St. Louis, MO) was used at a concentration of [50 μg/ml]. Melphalan was dissolved in diluent at a concentration of 50 mg/ml immediately before use. Etoposide (VP-16) (Bristol-Myers Squibb, New York, NY) was stored at a concentration of 33.98 mM and a final concentration of 50 μM was used in all experiments. Methotrexate (Sigma; [50 μg/ml]), vincristine (Sigma; [20 μg/ml]), docetaxel (Sigma; [50 μM]), carboplatin (Sigma; [50 μM]) or mechlorethamine hydrochloride (Sigma; [10 μM or 25 μM]) were included where indicated. KU-55933, an ATM kinase inhibitor (Calbiochem, Philadelphia, PA) was used at a concentration of 10 μM.

2.2. Elisa

BMSC and HOB were cultured in α-MEM complete media or complete osteoblast growth media until confluent, and subsequently treated with melphalan [50 μg/ml] for 24 h. Following treatment, the media was replaced, supernatants collected at 2, 4, 6, 8, 24, and 48 h post-treatment and the confluent layers of BMSC and HOB were lysed in RIPA buffer to allow quantitation of both supernatant and intracellular IL-6 protein levels. Following collection of samples at all timepoints, an IL-6 ELISA (eBioscience, San Diego, CA) was completed. In additional IL-6 ELISAs, BMSC and HOB were treated for 24 h with melphalan, VP-16, methotrexate, vincristine, docetaxel, carboplatin or mechlorethamine hydrochloride at the doses indicated previously. Following exposure, the cells were rinsed, media replaced and supernatants collected at 2, 4, 6, 8, 24 or 48 h post-treatment and an IL-6 ELISA completed. For ATM kinase inhibition experiments, BMSC or HOB were treated with KU-55933 [10 μM] or melphalan [50 μg/ml] for 24 h, rinsed and supernatants collected and IL-6 ELISA completed as described above. For co-culture experiments, BMSC and HOB were grown to confluence and H929 myeloma cells (ATCC: NCI-H929) were added at a density of 1×10^6 H929 cells per milliliter for 24 h prior to treatment with melphalan [50 μg/ml] for 24 h.

2.3. RNA isolation and real time PCR

Total RNA was isolated using the Qiagen RNeasy kit following the recommendations of the manufacturer (Qiagen Inc., Valencia, CA). RNA concentration was determined by NanoDrop. To determine relative levels of IL-6 expression, real-time PCR was completed. All reactions were performed in triplicate using 50 ng of RNA per reaction and the one-step QuantiTect SYBR Green RT-PCT kit (Applied Biosystems, Foster city, CA). Specific primers for IL-6 (Real Time Primers, Elkins Park, PA) or the housekeeping gene beta-glucuronidase (*GusB*) (Real Time Primers) were used. Amplifications were completed using a 7500 real-time cycler (Applied Biosystems). The amplification conditions included 50 °C/30 min, 95 °C/15 min and 45 cycles of 94 °C/15 s, 55 °C/30 s, and 72 °C/

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