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Bone marrow osteoblast vulnerability to chemotherapy

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Abstract

Osteoblasts are a major component of the bone marrow microenvironment which provide support for hematopoietic cell development. Functional disruption of any element of the bone marrow niche, including osteoblasts, can potentially impair hematopoiesis. We have studied the effect of two widely used drugs with different mechanisms of action, etoposide (VP16) and melphalan, on murine osteoblasts at distinct stages of maturation. VP16 and melphalan delayed maturation of preosteoblasts and altered CXCL12 protein levels, a key regulator of hematopoietic cell homing to the bone marrow. Sublethal concentrations of VP16 and melphalan also decreased the levels of several transcripts which contribute to the composition of the extracellular matrix (ECM) including osteopontin (OPN), osteocalcin (OCN) and collagen 1A1 (Col1a1). The impact of chemotherapy on message and protein levels for some targets was not always aligned, suggesting differential responses at the transcription and translation or protein stability levels. Since one of the main functions of a mature osteoblast is to synthesize ECM of a defined composition, disruption of the ratio of its components may be one mechanism by which chemotherapy affects the ability of osteoblasts to support hematopoietic recovery coincident with altered marrow architecture. Collectively, these observations suggest that the osteoblast compartment of the marrow hematopoietic niche is vulnerable to functional dysregulation by damage imposed by agents frequently used in clinical settings. Understanding the mechanistic underpinning of chemotherapy-induced changes on the hematopoietic support capacity of the marrow microenvironment may contribute to improved strategies to optimize patient recovery post-transplantation.

Keywords

bone marrow microenvironment; osteoblast; extracellular matrix; hematopoietic stem cell; chemotherapy

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Introduction

Hematopoietic stem (HSC) and progenitor cells require interaction with their niche in the bone marrow microenvironment for regulation of quiescence, cell cycle progression and differentiation [1–3]. Several types of cells in the bone marrow provide support to HSC and their lineage committed progenitors, including endothelial cells [4,5], CXCL12-abundant reticular cells (CAR) [6,7], nestin-positive mesenchymal stem cells (MSC) [8], CD146-positive subendothelial osteoprogenitors [9], and osteoblasts [10–12]. There is evidence to suggest that osteoblasts, as a defining component of the endosteal space, support HSC and regulate the fate of more differentiated hematopoietic progenitors [10,11]. Depletion of osteoblasts in mice profoundly affects hematopoiesis, manifested by decreases in HSC as well as lymphoid and myeloid progenitors [12], and B-cell commitment and maturation [13].

Osteoblasts are derived from MSCs through several somewhat poorly defined maturation stages. MSCs differentiate to osteoprogenitor cells through the activation of osteoblast-specific transcription factors such as Runx2 and subsequently Osterix (SP7) [14]. One of the earliest markers of preosteoblast cells is alkaline phosphatase (ALP) and its expression persists in subsequent maturation stages. Preosteoblasts also express Col1a1 protein, while at later stages they also start to produce OPN and further differentiate to mature osteoblasts which synthesize bone matrix [15]. These various markers provide tools to characterize specific stages of osteoblast differentiation.

In the current study we investigated how osteoblasts are affected by chemotherapy by utilizing an *in vitro* model to include drugs from two distinct classes used in clinical settings; Etoposide (VP16) which induces double strand DNA breaks by inhibition of topoisomerase II [16] and melphalan as an alkylating agent that damages DNA through crosslinking and the addition of adducts [17]. A number of chemotherapy drugs have been documented to functionally impair stromal cells in the bone marrow, including 1,3-bis(2-chloroethyl)-1-nitrosourea, busulfan, doxorubicin, VP16, methotrexate, and vincristine [18,19] suggesting their potential to impair hematopoietic support capacity. Bone density and colony forming unit fibroblasts (CFU-F) were shown to decrease in patients following allogeneic stem cell transplant [20]. Earlier work from our laboratory indicated that treatment of primary human osteoblasts with VP16 and melphalan activated the TGF- β 1 pathway [21], consistent with the finding that bone marrow stromal cells established from leukemia patients treated with chemotherapy have elevated levels of TGF- β 1 [22]. Chemotherapy exposure was also reported to affect osteoblast-specific proteins including type I collagen and alkaline phosphatase in human primary osteoblasts, as well as the ability of mature osteoblasts to mineralize bone [23].

In the current study we have demonstrated that chemotherapy exposure decreases expression of CXCL12, a key factor mediating homing and hematopoietic cell adhesion in the bone marrow niche, while also decreasing differentiation stage-specific synthesis of osteoblast components of the ECM including OCN, OPN and Col1a1. Treatment of preosteoblasts with VP16 or melphalan impaired their differentiation potential and decreased transcripts associated with osteoblast differentiation (Runx2, SP7, and OCN). VP16 and melphalan also altered hematopoietic cell support provided by osteoblasts, demonstrated by an increased proportion of Lin[–] Sca1⁺c-kit⁺ stem cells and an increased number of viable Sca1[–]c-kit⁺IL7R[–] myeloid progenitor cells following co-culture with chemotherapy damaged osteoblasts. Taken together, these data indicate that functional dysregulation of the osteoblast component of the bone marrow microenvironment might include both chemokine gradient changes as well as altered ECM deposition.

Materials and Methods

Cell lines, reagents and drug treatment

Murine pre-osteoblast cell line MC3T3E1, subclone 4, was purchased from ATCC (ATCC CRL-2593). Both MC3T3E1 and 7F2 cell lines were cultured in α -MEM supplemented with 10 % fetal bovine serum, 2 mM L-Glutamine, 1% sodium pyruvate, and penicillin/streptomycin, at 37°C in 6 % CO₂. VP16 (Bristol Myers Squibb, New York, NY) was used at 50–100 μ M for both MC3T3E1 and 7F2 cells; melphalan (Sigma) was dissolved in diluent containing 2% sodium citrate, 60 % Propylene Glycol, and 5.2 % EtOH, pH 1.1 immediately prior to use.

Differentiation of pre-osteoblast cells to mature osteoblasts

MC3T3E1 and 7F2 cells were plated in 24 well plates as confluent monolayers. To induce osteoblast differentiation medium was supplemented with 100 μ g/ml Ascorbic acid and 10 mM β -glycerol phosphate. Medium was exchanged every 3 days. 7F2 cells were assayed for differentiation after 7 days in culture and MC3T3E1 cells after 21 days. Cells were stained for alkaline phosphatase according to the manufacturer's protocol (SigmaFast BCIP/NBT kit or Leukocyte Alkaline Phosphatase kit, Sigma). Calcium deposition was monitored by Alizarin Red S staining as previously described [24].

Isolation of RNA and RT-PCR

RNA was isolated from osteoblasts using the RNeasy Mini kit with on-column DNase I digestion (Qiagen). One-step RT-PCR reactions were performed in triplicate using 50 ng of RNA per well, with the QuantiTect SYBR Green RT-PCR kit (Qiagen) on an Applied Biosystems 7500 Real Time PCR machine. GUS B, GAPDH or B2M were used as loading controls. The following primer sets were purchased from Invitrogen:

OCN	F: ACCCTGGCTGCGCTCTGTCTCT	R: GATGCGTTTGTAGGCGGTCTTCA
Runx2	F: TTTAGGGCGCATTCCTCATC	R: TGTCTTGTGGATTAAAGGACTTG
SP7	F: ACTCATCCCTATGGCTCGTG	R: GGTAGGGAGCTGGGTTAAGG
Col1a1	F: TGTGTGCGATGACGTGCAAT	R: GGGTCCCTCGACTCCTACA
PPARG2	F: TTTATGCTGTTATGGGTGAAACTC	R: AGAGGTCCACAGAGCTGATTCC
Adipoq	F: TGTTCTCTTAATCCTGCCCA	R: CCAACCTGCACAAGTTCCTT
CEBPA	F: TGGACAAGAACAGCAACGAG	R: TCACTGGTCAACTCCAGCAC
OPN	F: GTGAAAGTGACTGATTCTGGCA	R: TTTTCTTCAGAGGACACAGCATT

Primers for CXCL12, TGF- β 1, B2M, GUS B, BMP4, and GAPDH were purchased from Real Time Primers. Fold change was calculated by the $\Delta\Delta$ Ct method [25].

ELISA

ELISAs for CXCL12 α (R&D) and OPN (R&D) were performed according to the manufacturer's instructions. Cellular supernatants were evaluated, from osteoblasts that were either untreated, or treated for 24h with 100 μ M VP16 or 25 μ g/ml melphalan. For CXCL12 ELISAs medium was diluted 1:4 for 7F2 cells or used undiluted for MC3T3E1 cells. For OPN ELISA supernatants were diluted 1: 200.

Scanning electron microscopy (SEM)

Adult 20 week old Balb/c mice were treated either with VP16 diluent (65% polyethylene glycol 300, 8% Tween 80, 30% ethanol, 0.2% citric acid, and 3% benzyl alcohol) or 20 mg/kg VP16 once a day, for 72h. Twenty four hours after the final treatment the mice were sacrificed and marrow was dislodged by rinsing femurs with PBS at 37° C to expose the endosteal surface. Femurs were subsequently washed in 37° C PBS prior to immersion fixation in 1% formaldehyde, 2.5% glutaraldehyde in 0.15 M sodium cacodylate buffer pH 7.2 for 48 hours at 4° C. Samples were washed in sodium cacodylate buffer and post-fixed in 1% osmium tetroxide in the cacodylate buffer for 30 min. Bones were rapidly dehydrated in graded steps of acetone (25%–100%) and critically point dried using a Tousimis 815a Critical Point Dryer. Samples were mounted onto aluminum stubs and coated with a 40 nm-thick layer of Platinum using a Temescal BJD 200 E-Beam Evaporator. Samples were examined with a JEOL JSM-7600-F scanning electron microscope.

Isolation of murine hematopoietic stem and progenitor cells

Adult Balb/c mice were sacrificed with bone marrow collected from femurs and tibia. Bone marrow cells were labeled with biotinylated antibodies specific for CD5, CD45R, CD11b, Gr-1 (Ly6G/C), 7–4 and Ter119 according to the manufacturer's protocol (Lineage cell depletion kit, Miltenyi Biotec). Lineage-negative (Lin[−]) cells were isolated on an AutoMacs column using the Deplete S program (purity > 90 %). To assay the effect of drugs on the capacity of osteoblasts to support survival and differentiation of HSC and progenitor cells, 40,000 Lin[−] cells were co-cultured with a monolayer of MC3T3E1 osteoblasts (170,000 cells per 24-well) for 5 days in RPMI containing 10 % FBS and 10 ng/ml IL-3 (murine rIL-3, R&D Systems). MC3T3E1 were either untreated or pre-treated with 50 uM VP16 or 25 ug/ml melphalan for 24h and washed thoroughly before hematopoietic cells were added to the culture. Hematopoietic cells were collected at the termination of the experiment by collecting both the supernatant and the monolayer to include the hematopoietic cells attached to it. The two fractions of cells were combined and labeled with the following rat α - mouse antibodies (BD Biosciences): CD45-PE-Cy7, CD45R (B220)-Alexa Fluor 700, CD16/32-FITC, Sca1-APC, CD117-PerCP-Cy5.5 and CD127-PE. The viability of the cells was monitored by staining with Violet Live/Dead fixable dead cell stain (Invitrogen). The samples were run on a FACS Aria flow cytometer (BD Biosciences) and data were analyzed by FCS Express software (De Novo Software). Osteoblasts were excluded from analysis by gating out live/CD45[−] cells.

Statistical analysis

One-Way ANOVA with Holm-Sidak post hoc multiple comparison was performed using SigmaPlot Version 11.0 to detect differences in RT-PCR and ELISA data. Differences were considered statistically significant when p-value was < 0.05.

Results

To study how osteoblasts at varied differentiation stages react to drug exposure, we used two mouse pre-osteoblast cell lines, MC3T3E1 (subclone 4) and 7F2. 7F2 cells were clonally derived from p53^{−/−} mice, while MC3T3E1 are spontaneously immortalized mouse calvarial osteoblasts [26,27]. Both were reported to differentiate to mature osteoblasts in medium containing ascorbic acid and β -glycerol phosphate [26,27]. While both control and differentiated cells stained strongly for alkaline phosphatase, consistent with the appearance of strong ALP staining in early preosteoblast cells, only differentiated cells exhibited calcium deposits (Fig. 1 A, B). We further verified the differentiation to mature osteoblasts by monitoring the levels of OCN. Both 7F2 and MC3T3E1 cell lines exhibited induction of OCN mRNA with differentiation (Fig. 2). These observations suggest that the 7F2 and

MC3T3E1 cells faithfully recapitulate osteoblast differentiation and are an appropriate model for pre-osteoblast development.

To determine the effect of VP16 and melphalan on the ability of osteoblasts to support hematopoietic cells, we evaluated the changes in osteoblast-specific transcripts and several factors important for hematopoietic support. Based on the rationale of the question, clinically relevant drugs and sub-lethal concentrations were chosen for evaluation to allow investigation of functional alterations of the microenvironment. Importantly, no significant reduction in osteoblast viability was observed, evaluated by PrestoBlue, MTT assay, and Trypan blue, in cultures treated at the concentrations of drugs utilized in this study (data not shown). In MC3T3E1 cells we detected an approximate 5 fold increase in the transcript levels of OCN, both in undifferentiated and differentiated cells after VP16 treatment, while melphalan treatment increased OCN levels by 3 fold in undifferentiated cells and 1.5 fold in differentiated cells, respectively (Fig. 2A). OPN levels, which decrease with differentiation, were also elevated after VP16 and melphalan treatment in both control and differentiated MC3T3E1 cells. In contrast, the levels of Runx2, SP7 and Colla1 decreased in both undifferentiated and differentiated MC3T3E1 cells with treatment. RunX2, SP7, and Colla1 were affected similarly in 7F2 cells (Fig. 2B). For 7F2 cells, OCN levels were affected differently by VP16 and melphalan. While VP16 induced an increase in OCN transcript levels similar to MC3T3E1 cells, melphalan treatment resulted in decreased OCN levels.

We also evaluated the effect of VP16 and melphalan on the mRNA levels of CXCL12 and BMP4 (Fig. 2 and data not shown). The level BMP4 transcript was not affected by either differentiation or treatment with VP16 or melphalan in both 7F2 and MC3T3E1 cells (data not shown). VP16 treatment resulted in an approximate 2 fold increase in CXCL12, while melphalan decreased CXCL12 transcript levels, by 7.7 fold and 5.6 fold in undifferentiated and differentiated MC3T3E1 cells, respectively (Fig. 2A). 7F2 cells exhibited a decrease in CXCL12 after treatment with either VP16 or melphalan (Fig. 2B).

To investigate the changes in CXCL12 and OPN at a protein level, ELISA of culture supernatants was performed (Fig. 3). CXCL12 and OPN protein response following treatment differed from the transcriptional responses shown in Fig. 2. Secreted CXCL12 levels were significantly lower than untreated controls in undifferentiated and differentiated cells for both MC3T3E1 and 7F2 (Fig. 3 A and B). OPN was affected similarly in MC3T3E1 and 7F2 as well, showing a decrease in undifferentiated cells treated with melphalan and also differentiated compared to undifferentiated controls (Fig. 3C and data not shown). Since sublethal doses of chemotherapy altered the composition of extracellular matrix proteins and delayed osteoblast maturation we sought to determine what effect these agents had on the ultrastructure of the endosteum. To observe gross morphology of the endosteum following treatment with VP16 *in vivo* we performed SEM on diluent controls (Fig. 4, A and B) and VP16 treated (Fig. 4, C and D) long bones. The endosteal surface is composed of cord/rope like structures covered by a smooth surface coat (arrows) in diluent controls. Lacunae vary in size and number but were found to be present over the entire endosteal surface (Fig. 3A). In addition, smooth electron dense patches (*, Fig. 4A) were also identified on the endosteal surface (Fig. 3B). Overall, the endosteal surface was intact and exhibited a uniform surface coat. VP16 treatment caused disruption of the endosteal surface lining (Fig. 4C). Material composing the electron dense patches condensed into clumps (*, Fig. 4C) exposing the underlying matrix. The surface coat covering the cord/rope structures was gone (Fig. 4D) exposing collagen fibers (arrows, inset, Fig. 4D). The appearance of the endosteum following treatment with VP16 provides only a general visual representation of the damage caused by VP16 in the osteoblastic niche but further characterization is required to come to specific conclusions beyond that.

We next determined whether treatment of 7F2 preosteoblasts with VP16 or melphalan would affect their ability to differentiate to mature osteoblasts, utilizing ALP as an indicator. Overall staining for ALP was reduced by drug treatment in cells cultured in both control and differentiation medium (Fig. 5B). At the same time, microscopic examination of the cultures revealed a population of cells staining intensely for ALP (Fig. 5A), which would correspond to cells at an earlier stage of osteoblast differentiation. We further determined the OCN, Runx2, SP7, Col1a1 and CXCL12 transcript levels of cells which were treated with drugs and then allowed to differentiate for 7 days (Fig. 5C). CXCL12 mRNA abundance did not change with differentiation or after drug treatment, but OCN mRNA decreased approximately 20 fold after VP16 exposure and 10 fold after melphalan exposure. Similarly, a statistically significant decrease in transcript levels was detected for RUNX2, SP7 and Col1a1. These data are consistent with delayed osteoblast maturation after exposure to VP16 and melphalan. MC3T3E1 cells had similar results (data not shown).

Both 7F2 cells and MC3T3E1 cells treated with VP16 or melphalan exhibited vacuoles in the cells, reminiscent of lipid droplets. Based on reports of adipocyte differentiation after chemotherapy treatment of osteoblasts [28] we stained the cultures for the presence of lipids with Oil Red and confirmed that the observed vacuoles indeed contained lipids (Fig. 5D, top and data not shown). However, there was no upregulation of adipocyte-specific transcripts and instead PPARG2, CEBPA, and Adipoq decreased significantly after drug treatment of 7F2 cells, both in control and differentiation medium (Fig. 5E, bottom). Thus, it is unlikely that drug-treated cells, in this model, undergo adipocyte differentiation.

We next determined the effect of VP16 and melphalan on the ability of MC3T3E1 cells to support HSC utilizing Lineage-negative cells co-cultured with a monolayer of MC3T3E1 cells (untreated or treated with VP16 or melphalan as described in materials and methods). To follow HSC support *in vivo*, we observed Lin[−]Sca1⁺c-kit⁺ cells (LSK), a cellular subset which would mark about 25 % of the hematopoietic stem/progenitor cells (HSPC) in Balb/c mice [29]. In the Lin[−] fraction of cells we determined the relative percentage of HSPCs, myeloid progenitors (Sca1[−]c-kit⁺IL7R[−]), lymphoid progenitors (Sca1⁺c-kit⁺IL7R⁺), as well as mature myeloid cells (CD16/CD32⁺) at the day of plating and after 5 days in culture with chemotherapy pre-treated or matched untreated control MC3T3E1 cells. After 5 days in culture about 80% of the Lin[−] cells incubated in media alone were viable, while Lin[−] cells in co-culture with untreated “healthy” osteoblasts were approximately 94% viable (Fig. 6A). Osteoblasts pre-treated with either VP16 or melphalan also supported the Lin[−] cell survival to a degree higher than media alone. Hematopoietic cell death in co-cultures of chemotherapy pre-treated osteoblasts was noted to be slightly higher than in cultures with control osteoblasts, at approximately 8.5% and 12.0%, respectively, compared to 6% for untreated osteoblasts. Osteoblasts generally supported differentiation of Lin[−] cells to CD16/CD32⁺ mature myeloid cells (Fig. 6B). Melphalan-treated osteoblasts supported marginally less myeloid cell differentiation compared to untreated cells (88.8% vs. 95.3%), while VP16 had no effect on MC3T3E1 ability to support myeloid differentiation.

Because the Lin[−] compartment contains not only stem cells but also committed hematopoietic progenitors, we further evaluated the percentage of LSK cells which are IL7R⁺ (lymphoid progenitors) and the Lin[−]Sca1[−]c-kit⁺IL7R[−] as myeloid progenitors in contrast to mature CD16/CD32⁺ myeloid cells noted earlier [30]. We detected a higher percentage of both lymphoid and myeloid progenitors co-cultured with VP16 or melphalan pre-treated osteoblasts compared to untreated osteoblast layers after 5 days in culture (Fig. 6 B and D). Our results indicate that VP16 and melphalan modulate the capacity of osteoblasts to support hematopoiesis and alter the ratio of stem and progenitor cells in this *in vitro* model, although the quantitative differences were modest.

Discussion

The current study focused on the effects of VP16 and melphalan on osteoblast cells at different stages of maturation. Cell lines utilized have been confirmed to be able to differentiate to mature osteoblasts by calcium deposition detected by Alizarin Red staining and upregulation of osteocalcin (Fig. 1). Both preosteoblast cells and mature osteoblasts expressed ALP which is consistent with its onset of detection in early preosteoblast cells [31]. We also detected similar amounts of osteoblast-specific transcription factors Runx2 and SP7 in preosteoblasts and mature osteoblasts, consistent with the MC3T3E1 and 7F2 cells being downstream of the MSC stage [14]. Collectively these observations suggested a reasonable *in vitro* model in which to evaluate whether chemotherapy vulnerability varies with stage of differentiation.

VP16 and melphalan altered the expression of several osteoblast-specific transcripts (Fig. 2). The Runx2 and SP7 transcription factors, and Col1a1 were uniformly downregulated in both MC3T3E1 and 7F2 cells regardless of differentiation stage following treatment. On the other hand, OCN is differentially regulated in 7F2 and MC3T3E1 cells; treatment with drugs increases OCN levels in MC3T3E1 cells, while melphalan decreases OCN levels in 7F2 cells. This might result from different basal OCN levels in 7F2 and MC3T3E1 cells, since the lack of p53 in 7F2 cells contributes to faster growth and osteoblast differentiation in this cell line. Since Col1a1 is a major component of the ECM, and OCN and OPN are immobilized on the ECM, these chemotherapy drugs have the potential of altering the adhesion properties of the ECM of both pre- and mature osteoblasts. Consistent with this, we detected disruption of the integrity of the “architecture” of the endosteal region of femurs from VP16-treated mice by SEM (Fig. 4). More specific analyses will be required to characterize the exact anatomical alterations, and the scope of specific cellular elements that are impacted on, but the observation is consistent with a general vulnerability of this anatomical site to chemotherapy induced stress. Melphalan exposure yielded similar results (data not shown).

We have also observed that VP16 and melphalan impair factors (including CXCL12 and OPN) which are important for hematopoietic cell differentiation (Figs. 2 and 3). We observed that although VP16 treatment of MC3T3E1 cells increased CXCL12 transcripts, secreted protein was decreased in both 7F2 and MC3T3E1 with treatment of either drug relative to untreated controls (Fig. 2 and 3). The differences in CXCL12 transcriptional regulation between 7F2 and MC3T3E1 might be linked to the fact that the cell lines have different basal expressions of CXCL12, produced at much higher levels in 7F2 cells. Again, this could be explained by the fact that 7F2 cells lack p53, which has been reported to downregulate secreted CXCL12 in fibroblast cells [32]. Our results are also consistent with a previous report that in human osteoblasts CXCL12 levels are sustainably reduced following treatment with VP16 and melphalan, resulting in diminished adhesion of CXCR4+ hematopoietic cells [21]. An additional report has linked the absence of CXCL12 with HSC quiescence, but also with increased myeloid differentiation [7]. Since in this model CXCL12 was reduced by ablation of CXCL12 abundant reticular cells, which also secrete other cytokines and factors important for HSC [7], we feel that the distinct observations related to the role of CXCL12 in hematopoietic cell support could be explained by differences in the experimental settings and interplay between several factors.

We further evaluated the levels of OPN protein and detected a decrease after melphalan treatment in undifferentiated MC3T3E1 cells (Fig. 3). It has been shown that OPN inhibits HSC proliferation and reduces differentiation of HSCs to myeloid cells *in vitro* [33]. We have also detected altered Lin[−] support and hematopoietic cell differentiation of chemotherapy pre-treated MC3T3E1 cells (Fig. 6), consistent with the above observation.

Deregulation of OPN in our model provides a potential mechanism by which hematopoiesis can be altered by chemotherapeutics.

Finally, we determined that VP16 and melphalan also disrupt the ability of preosteoblast cells to differentiate to mature osteoblasts. Drug treatment induced delayed osteoblast maturation, as evidenced by a significant decrease in a number of transcripts required for establishment of the mature osteoblast, including OCN, Runx2, SP7, and Col1a1. We also detected the appearance of a population of cells staining intensely for ALP, consistent with ALP being expressed at the highest level in preosteoblast cells (Fig. 4). ALP increases, and Col1a1 decreases, have been noted before in primary human osteoblasts from patients undergoing chemotherapy with various drugs [23], and a preferential drug effect on viability of preosteoblast cell lines compared to cultures containing mature osteoblasts was also reported [19]. Additionally, VP16 and melphalan treated osteoblasts exhibited increased lipid content but no coincident upregulation of adipocyte-specific markers (Fig. 4D and E). Increased lipid staining after *in vivo* treatment with 5-fluorouracil has been interpreted before to indicate preferential adipocyte differentiation of murine CXCL12-expressing cells [6], but we did not see a correlation between the lipid increase and appearance of adipocyte-specific differentiation markers. Thus, the increased lipid content of the chemotherapy treated osteoblasts could be explained by an alternative mechanism, with one possibility being autophagosome formation. Results from the current study contribute to better understanding the scope of effects of genotoxic stress and DNA damage on the bone marrow microenvironment. DNA double strand breaks have been shown to promote cell differentiation in normal B-cell development [34], in neuronal stem and progenitor cells [35], and in melanocyte stem cells [36]. Conversely, DNA damage impairs proper differentiation of myoblasts when applied before induction of differentiation [37]. Our data in a model of pre- and mature osteoblasts are consistent with the assertion that DNA damage can influence differentiation in a component of the marrow microenvironment. This damage is also associated with alteration of ECM gene expression profiles, disrupted structural integrity, and dysregulated support of HSC differentiation. A better understanding of the diversity of vulnerability of populations of cells in the marrow to drugs with distinct mechanisms of action may support improved approaches to pre-transplant conditioning and subsequent restoration of steady state hematopoiesis.

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Figure 1.

Differentiation of MC3T3E1 and 7F2 cells to mature osteoblasts.

7F2 (A) or MC3T3E1 (B) cells were incubated either with regular growth medium (undifferentiated) or osteoblast differentiation medium (differentiated) for 7 days (7F2 cells) or 21 days (MC3T3E1 cells), respectively. Cells were stained for Alkaline phosphatase (ALP) and Alizarin Red as a measure of osteoblast differentiation.

Figure 2.

Effect of VP16 and melphalan on the expression of osteoblast-specific transcripts. MC3T3E1 cells were incubated either in regular growth medium (undifferentiated) or osteoblast differentiation medium (differentiated) for 21 days, after which the cells were treated with 100 μ M VP16 or 25 μ g/ml melphalan (A). Total RNA was isolated and qPCR was performed with primers specific for OCN, Runx2, SP7, Col1a1, OPN and CXCL12. Relative expression was normalized to the expression in undifferentiated untreated cells (undifferentiated untr). (B) 7F2 cells were differentiated for 7 days and treated with 100 μ M VP16 or 25 μ g/ml melphalan (B) and then processed as described for (A). Error bars denote standard deviation from triplicate measurements in a representative experiment. * indicates significantly greater or lesser than untreated control (for drug treatments) or undifferentiated untreated control, p-value < 0.05, One-Way ANOVA.

Figure 3.

Effect of VP16 and melphalan on the levels of secreted CXCL12 and OPN. MC3T3E1 cells were incubated either in regular growth medium (undifferentiated) or osteoblast differentiation medium (differentiated) for 21 days, after which medium was removed and replaced with fresh medium (untreated) or medium containing 100 μ M VP16 or 25 μ g/ml melphalan. After 24 hours, medium was removed and ELISAs were performed for CXCL12 (A) and OPN (C). 7F2 cells were differentiated for 7 days then treated as in A prior to ELISA for CXCL12 (B). * indicates significantly greater or lesser than untreated control (for drug treatments) or undifferentiated untreated control, p-value < 0.05, One-Way ANOVA, n=3.

Figure 4.

Scanning electron microscopy of murine bone. Representative scanning electron micrographs of mouse control and VP16-treated long bones. In diluent-treated controls (A, B) the endosteum consists of an intact uniform surface coat covering the underlying ECM. (B). Enlarged view of smooth electron dense patches (*, Fig. 3A) found dispersed throughout the endosteum. VP16 treatment (C, D) caused disruption of the endosteal surface coat (*) exposing the underlying extracellular fibrillar matrix (arrows inset). Bar, 10 μ M.

Figure 5. Effect of VP16 and melphalan on the differentiation potential of preosteoblast cells 7F2 cells were exposed for 24 h to 50 uM VP16, 25 ug/ml melphalan, VP16 diluent (VP16 dil) or melphalan diluent (Mel dil), respectively. Following treatment cells were washed and incubated with regular growth medium (undifferentiated) or osteoblast differentiation medium (differentiated) for 7 days. Cells were stained for Alkaline phosphatase (ALP) and images were taken with (A) or without (B) 20x magnification. (C). Total RNA was isolated from cells treated as in A and qPCR was performed with primers specific for OCN, Runx2, SP7, Col1a1 and CXCL12. Relative expression of the genes was normalized to the expression in untreated undifferentiated cells. (D). Oil Red staining for lipids in cells treated as in (A). Real time PCR for the adipocyte-specific transcripts PPARG2, CEBPA, and Adipoq (E). Error bars denote standard deviation from triplicate measurements. * indicates significantly greater or lesser than untreated control (for drug treatments) or undifferentiated untreated control, p-value < 0.05, One-Way ANOVA.

Figure 6.

Support of murine hematopoietic stem and progenitor cells by osteoblasts treated with VP16 or melphalan. MC3T3E1 cells were seeded as monolayers in 24-well plates. Cells were treated with 50 μ M VP16, 25 μ g/ml melphalan, or were left untreated for 24h. After several washes of the osteoblasts, 40,000 freshly isolated lineage negative cells were added to the osteoblasts in RPMI medium containing 10 % FBS and 10 ng/ml IL-3. Cultures were incubated for 5 days, after which cells were trypsinized and stained with Violet Live/Dead fixable dead cell stain and antibodies to CD45, Sca-1, c-kit, CD16/32, B220 and CD127. Number of dead cells (A), Sca+c-kit+ (LSK) and lymphoid progenitors (CLP) (C), myeloid progenitors (D) and CD16/CD32+ myeloid cells (B) was determined by flow cytometry.